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Compartmentalization and transport in β-lactam antibiotic biosynthesis by filamentous fungi

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Key words: cephalosporin, cephamycin, glutathione, microbody, organelle, penicillin, peptide antibiotics, plasma membrane, peroxisome, transport, vacuole, *Aspergillus nidulans*, *Cephalosporium acremonium*, *Penicillium chrysogenum*

Abstract

A proper description of the biosynthesis of fungal β-lactam antibiotics requires detailed knowledge of the cell biology of the producing organisms. This involves a delineation of the compartmentalization of the biosynthetic pathways, and of the consequential transport steps across the cell-boundary plasma membrane and across organellar membranes. Of the enzymes of the penicillin biosynthetic pathway in *Penicillium chrysogenum* and *Aspergillus nidulans*, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and isopenicillin N synthase (IPNS) probably have a cytosolic location. Acyl-coenzyme A:isopenicillin N acyltransferase (IAT) is located in microbodies. Of the two enzymes that may be involved in activation of the side chain, acetyl-coenzyme A synthetase (ACS) is located in the cytosol, and phenylacetyl-coenzyme A ligase (PCL) is probably located in the microbody. All enzymes of the cephalosporin biosynthesis pathway in *Cephalosporium acremonium* probably have a cytosolic location. The vacuole may play an ancillary role in the supply of precursor amino acids, and in the storage of intermediates. The distribution of precursors, intermediates, end- and side-products, the transport of nutrients, precursors, intermediates and products across the plasma membrane, and the transport of small solutes across organellar membranes, is discussed. The relevance of compartmentalization is considered against the background of recent biotechnological innovations of fungal β-lactam biosynthesis pathways.

Abbreviations: *A. nidulans – Aspergillus nidulans*; *A. niger – Aspergillus niger*; Aad – α-aminoadipic acid; ACS – acetyl-CoA synthetase; ACV – δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine; ACVS – ACV synthetase; ACA – aminocephalosporanic acid; ADAC – aminodeacetylcephalosporanic acid; ADCA – aminodeacetoxycephalosporanic acid; Adi – adipic acid; 6-APA – 6-aminopenicillanic acid; *bis*-ACV – oxidised ACV; *C. acremonium – Cephalosporium acremonium*; Ceph – cephalosporin; CoA – coenzyme A; DAC – deacetylcephalosporin C; DACS – DAC synthase; DAOC – deacetoxycephalosporin C; DAOCS – DAOC synthase; DAT – acetyl-CoA:DAC acetyltransferase; *E. coli – Escherichia coli*; IAT – acyl-CoA:IPN acyltransferase; γGT – γ-glutamyltranspeptidase; γGCT – γ-glutamylcyclotransferase; GSH – glutathione; GSHred – GSH disulphide reductase; GST – GSH S-transferase; GSX – glutathione S-conjugate; GSSG – oxidised GSH; 8-HPA – 8-hydroxypenillic acid; IPN – isopenicillin N; IPNS – IPN synthase; αKG – α-ketoglutarate; MFS – major facilitator superfamily; *N. crassa – Neurospora crassa*; *N. lactamdurans – Nocardia lactamdurans*; 5OP – 5-oxoprolinase; OPC – 6-oxopiperidine-2-carboxylic acid; PA – phenylacetic acid; *P. chrysogenum – Penicillium chrysogenum*; PCL – PA-CoA ligase; Pen – penicillin; *pmf* – proton motive force; POA – phenoxy acetic acid; *P. putida – Pseudomonas putida*; *S. cerevisiae – Saccharomyces cerevisiae*; *S. clavuligerus – Streptomyces clavuligerus*; *S. lipmanii – Streptomyces lipmanii*; TrxAB – broad-range thioredoxin-dependent disulphide reductase.

1. Introduction

Compartmentalization of metabolic reactions plays an important role in the regulation and determination of metabolic fluxes. Amongst others, it creates a defined environment for enzymes to act in, and enables the establishment of specific substrate concentrations that are sufficiently high for enzymes to act upon. On the other hand, it imposes the need to import substrates or their precursors, and to export products if they have to fulfil their function outside the cell or the compartment in which they are synthesized. The simple level of compartmentalization which is encountered in the bacterial cell, namely, the cytoplasm surrounded by a single or a double membrane, is extensively elaborated upon in fungi. The fungal cell, which is separated from its environment by the plasma membrane surrounded by a cell wall, additionally contains endogenous membranes. These membranes encompass a diversity of organelles, including nuclei, endoplasmatic reticulum, mitochondria, vacuoles, microbodies, Woronin bodies, and traffic vesicles (Markham, 1995; Pringle et al., 1997).

It has been well recognised that significant parts of primary metabolic routes are compartmentalized in the fungal cell (Griffin, 1994; Gow & Gadd, 1995). However, less is known about the contribution of compartmentalization to secondary metabolism. The relatively well-studied pathway of penicillin (Pen) biosynthesis by the filamentous fungus *Penicillium chrysogenum* (*P. chrysogenum*) represents such a secondary metabolic route. Recently, it has become clear that Pen biosynthesis partly takes place inside a microbody-like organelle (Müller et al., 1991,1992), and that the vacuole is involved as well (Lendenfeld et al., 1993). In this review, an up-to-date description will be given of the compartmentalization of the biosynthetic pathways for β-lactam antibiotics by filamentous fungi, and of the consequential transport steps. Considered are the antibiotics Pen, which is produced by *P. chrysogenum* and *Aspergillus (Emericella) nidulans* (*A. nidulans*), and cephalosporin (Ceph), which is produced by *Cephalosporium acremonium* (*C. acremonium*; also named *Acremonium chrysogenum*). For reviews and monographs on different aspects of fungal β-lactam antibiotic production, see, e.g., Herschbach et al. (1984), Swartz (1985), Smith (1985), Martín & Liras (1989), Kleinkauf & von Döhren (1991), Martín et al. (1991), Aharonowitz et al. (1992), Brakhage & Turner (1995), Martín & Gutíerrez (1995), Nielsen (1995), Martín et al. (1997), Brakhage (1998), and Schügerl (1998). Compartmentalization of β-lactam biosynthesis steps may have special retributions to industrially interesting processes which involve heterologous expression of genes. A few examples that highlight this point, will be discussed.

2. Location of enzymes involved in β-lactam antibiotic biosynthesis

The Pen biosynthetic pathway involves the catalytic action of three enzymes, viz. δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (ACVS), isopenicillin N (IPN) synthase (IPNS) and acyl-coenzyme A:isopenicillin N (acyl-CoA:IPN) acyltransferase (IAT) (Fig. 1). The location of these enzymes has been best studied for *P. chrysogenum*, and will be discussed below. Ceph biosynthesis by *C. acremonium* involves an additional set of enzymes, viz. the bifunctional deacetoxycephalosporin (DAOC) synthase / deacetylcephalosporin (DAC) synthase (DAOCS/DACS; expandase/hydroxylase), and acetyl-CoA:DAC acetyltransferase (DAT) (Fig. 1). Their location, and the location of some auxilliary enzymes that are relevant for β-lactam biosynthesis, will be discussed as well. Models depicting the putative compartmentalization of the pathways for Pen and Ceph biosynthesis are presented in Fig. 2, alongside with the situation for cephamycin biosynthesis by the Gram-positive bacterium *Streptomyces clavuligerus* (*S. clavuligerus*) (see also Fig. 1).

2.1. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (ACVS)

The peptide synthetase ACVS (Table 1) catalyses the first step in both Pen and Ceph biosynthesis, in which the ACV tripeptide precursor is formed (Zhang & Demain, 1992a; Aharonowitz et al., 1993a; Kleinkauf & von Döhren, 1996) (Fig. 1). ACVS is a multi-enzyme with an unconventional large size and complex domain structure. These properties are reflected in its instability and sedimentation properties, which make it particularly difficult to investigate (Zhang & Demain, 1992a). Reports concerning a membrane-confined or -attached location of ACVS from *P. chrysogenum* have dominated the earlier literature. For example, Abraham and co-workers have described that ACVS activity is associated with a particulate fraction (Fawcett & Abraham, 1975,1976; Abraham et al., 1981). By measuring ACVS activity in subcellular fractions,

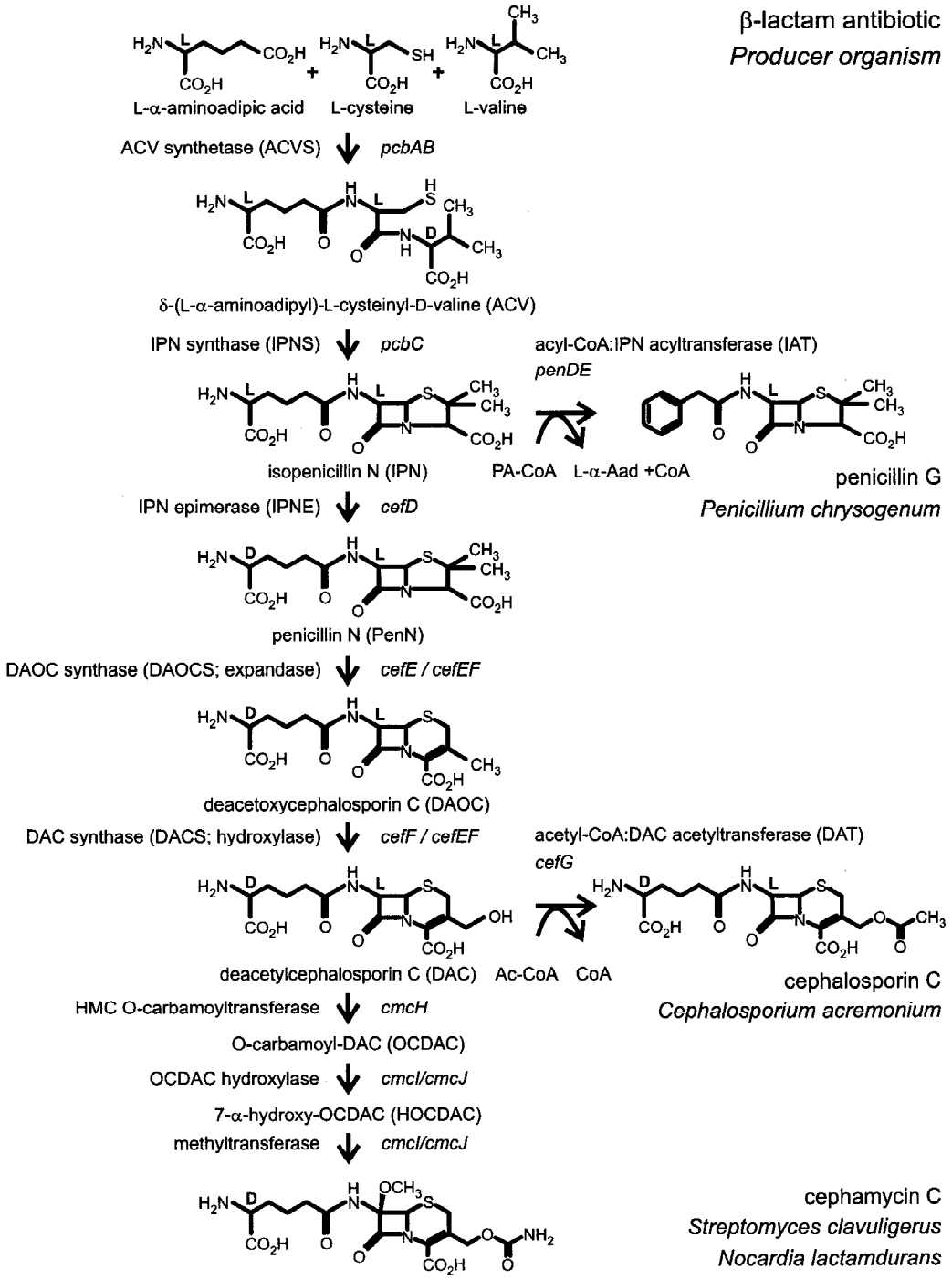

Figure 1. Biosynthetic pathways for the fungal β-lactam antibiotics penicillin G, produced by *Penicillium chrysogenum* and *Aspergillus nidulans*, and cephalosporin C, produced by *Cephalosporium acremonium*, and for the bacterial β-lactam antibiotic cephamycin C, produced by *Streptomyces clavuligerus* and *Nocardia lactamdurans*. Gene names are denoted in italics.

Table 1. Genes, enzymes, and their characteristics (size, affinities, pH optimum, and location), involved in fungal β-lactam antibiotic biosynthesis, and corresponding genes and enzymes involved in bacterial β-lactam antibiotic biosynthesis

Protein[a]	Org.[b]	Gene[c]	Protein size		Ref	K_m[d]	pH opt	Ref	Compart-ment[e]	Ref
			aa res	kDa		μM				
ACVS	*A.nid*	*acvA*	3770	422.5	Mc91a	nd	nd	vL89	cytosol (def)	
	C.acr.	*pcbAB*	3712	414.8	Gu91a	170 (aad), 26 (Cys), 340 (Val)	7.5	Ba87, Ba90	cytosol (def)	
						120 (Aad), 90 (Cys), 320 (val)	8.3	Ka98		
	P.chr.	*acvA*	3776	424.0	Sm90b,Br95	46 (Aad), 80 (Cys), 83 (Val)	8.4	Th97	vacuole/	Le93/
		pcbAB	3792	426.0	Di90				cytosol	vdK98
	N.lac.	*pcbAB*	3649	404.1	Co91	nd	nd	Co96a	cytosol (def)	
	S.cla.	*pcbAB*	nd	nd	To91,Je93	560 (Aad), 70 (Cys), 1140 (Val)		Je88	cytosol (def)	
						630 (Aad), 120 (Cys), 300 (Val)	8.5	Zh92		
						630 (Aad), 430 (Cys), 380 (Val)		Ka95		
IPNS	*A.nid*	*IPNS*	331	37.5	Ra87	nd	nd		cytosol (def)	
	C.acr.	*IPS*	338	38.4	Sa85	170 (ACV)	nd	Bal85ab, 87	cytosol (def)	
						300 (ACV)		Ku83, Pa84		
	P.chr.	*IPS*	331	38.0	Ca86	130 (ACV)	7.8	Ra85	cytosol	Mu91
		ips	331	38.0	Ba89a					
	N.lac	*pcbC*	328	37.5	Co91	180 (ACV)	nd	Ca88	cytosol (def)	
	S.cla.	*IPNS*	329	36.9	Le88	320 (ACV)	7.0	Je86	cytosol (def)	
IAT	*A.nid.*	*penDE*	357	39.2	Mo90, To90	nd	nd		microbody (seq)	
			102+255	11.4+27.8						
	P.chr.	*aat*	357	39.9	Ba89b,To90	4000 (IPN), 23 (IPN+PA-CoA)	8.0-8.5	Al87,93	microbody	Mu91,92
			102+255	11.5+285		9.3 (6-APA+PA-CoA), 6 (PA-COA), 2000 (PenV)				
IPNE	*C.acr.*	nd				nd	nd		cytosol (def)	
	N.lac.	*cefD*	398	43.6	Co93b	270 (IPN)	7.0	La90	cytosol (def)	
	S.cla.	*cefD*	398	43.5	Ko90	nd	nd	Us89	cytosol (def)	

Table 1. Continued.

DAOCS /DACS	*C.acr.*	*cefEF*	332	36.5	Sa87	29 (PenN), 22 (αKG) /18 (DAOC), 20 (αKG)	7.5-7.8 7.0-7.5	Ye91	cytosol (def)
DAOCS	*N.lac.*	*cefE*	314	34.5	Co93b	52 (PenN), 3 (αKG)	(5-11)	Co87	cytosol (def)
	S.cla.	*cefE*	311	34.5	Ko89	35 (PenN), 22(αKG)	7.4	Ye91	cytosol (def)
DACS	*N.lac.*	*cefE*	311	34.4	Co96b	nd	nd		cytosol (def)
	S.cla.	*cefF*	318	34.6	Ko91	25 (DAOC), 14 (αKG)	7.0-7.4	Ye91	cytosol (def)
DAT	*C.acr.*	*cefG*	444	49.3	Gu92	nd	7.0-7.5	Fu75,Fe80	cytosol (def)
			385		Ma92				
			399	41.0	Ma93				
ACS	*P.chr.*	*acuA*	669	74.3	M93	6800 (AcOH), 180 (CoA),	8.0	Ma92	cytosol (def)
		facA	669	74.3	Go93	17000 (ATP)			
PCL	*P.chr.*	*pcl*	578	63	WO97	nd	9.0	WO97	microbody (seq)
	P.put.	*pcl*	438	48	Mi98	16600 (PA)	7.0-7.4	Ma90,Lu95	cytosol (def)
TrxAB	*P.chr.*	*trxA*	106	11.3	Co94	125 (*bis*-ACV) 800 (GSSG)	nd	Co94	cytosol (def)
		trxB	334	35.6					
GSHred	*P.chr.*	nd				nd	nd	Co94	cytosol (def)
TrxAB	*S.cla.*	nd		12.5	Ah93b	210 (*bis*-ACV) 310 (GSSG)	nd	Ah93b	cytosol (def)
		nd		34.5					

[a]Proteins: for abbreviations, see Abbreviations. [b]Organisms: *A.nid.*, *A. nidulans*; *C.acr.*, *Cephalosporium acremonium* (= *Acremonium chrysogenum*); *P.chr.*, *P. chrysogenum*; *N.lac.*, *N. lactamdurans*; *S.cla.*, *S. clavuligerus*; *P.put.*, *P. putida*; and see Abbreviations. [c]Genes: consensus names for the genes of penicillin and cephalosoprin biosynthesis are *pcbAB (acvA*), *pcbC* (*ipnA*), *penDE* (*aat* or *aatA*), *cefD*, *cefEF*, and *cefG*. [d]Affinity constants; for substrates, see Abbreviations. (Note that some enzymes bind other substrates or co-factors as well, such as ATP, O_2, or Fe, but these are not considered here.) [e]Compartment; (def), default (cytosolic in the absence of indications from the sequence or biochemical data); (seq), based on sequence data.

References: Ah93b, Aharonowitz et al. 1993b; Al87, Alvarez et al. 1987; Al93, Alvarez et al. 1993; Ba87, Banko et al. 1987; Bal85a, Baldwin et al. 1985a; Bal85b, Baldwin et al. 1985b; Bal87, Baldwin et al. 1987; Ba89a, Barredo et al. 1989a; Ba89b, Barredo et al. 1989b; Ba90, Baldwin et al. 1990; Br95, Brakhage & Turner 1995; Ca86, Carr et al. 1986; Ca88, Castro et al. 1988; Co87, Cortés et al. 1987; Co91, Coque et al. 1991; Co93b, Coque et al. 1993b; Co94, Cohen et al. 1994; Co96a, Coque et al. 1996a; Co96b, Coque et al. 1996b; Di90, Díez et al. 1990; Fe80, Félix et al. 1980; Fu75, Fujisawa & Kanzaki 1975; Go93, Gouka et al. 1993; Gu91a, Gutiérrez et al. 1991a; Gu92, Gutiérrez et al. 1992; Je86, Jensen et al. 1986; Je88, Jensen et al. 1988; Je93, Jensen et al. 1993; Ka95, Kadima et al. 1995; Ka98, Kallow et al. 1998; Ko89, Kovacevic et al. 1989; Ko90, Kovacevic et al. 1990; Ko91, Kovacevic & Miller 1991; Ku83, Kupka et al. 1983; La90, Laíz et al. 1990; Le88, Leskiw et al. 1988; Le93, Lendenfeld et al. 1993; Lu95, Luengo 1995; Ma90, Martínez-Blanco et al. 1990; Ma92, Matsudo et al. 1992; Ma93, Mathison et al. 1993; Mc91a, MacCabe et al. 1991a; Mi96, Miñambres et al. 1996; Mo90, Montenegro et al. 1990; Mu91, Müller et al. 1991; Mu92, Müller et al. 1992; Pa84, Pang et al. 1984; Ra85, Ramos et al. 1985; Ra87, Ramón et al. 1987; Sa85, Samson et al. 1985; Sa87, Samson et al. 1987; Sm90b, Smith et al. 1990b; Th97, Theilgaard et al. 1997; To90, Tobin et al. 1990; To91, Tobin et al. 1991; Us89, Usui & Yu 1989; vdKa, van de Kamp et al. unpublished; vL89, van Liempt et al. 1989; WO97, International patent WO97/02349; Ye91, Yeh et al. 1991; Zh92, Zhang et al. 1992.

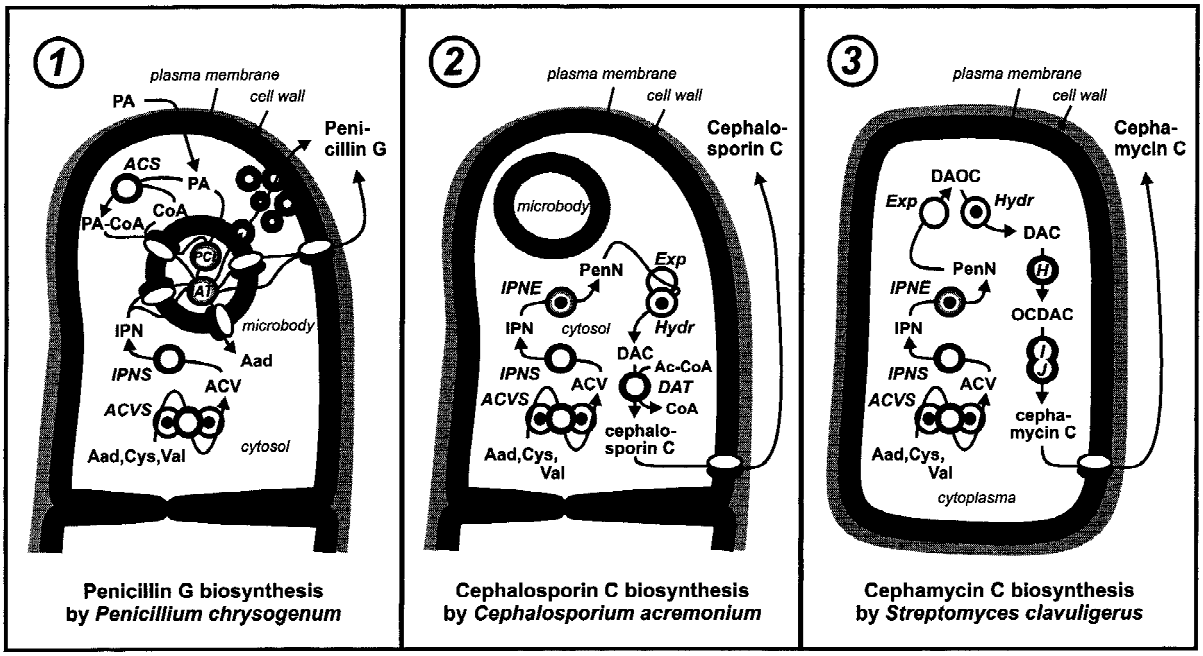

Figure 2. Model describing the compartmentalization of the penicillin G and cephalosporin C biosynthetic pathways in the filamentous fungi *Penicillium chrysogenum* (1), and *Cephalosporium acremonium* (2). For comparison, the 'compartmentalization' of the cephamycin C biosynthetic pathway in *Streptomyces clavuligerus* is shown (3). Putative transport steps are discussed in the text. Some putative enzymatic and transport steps are depicted as parallel routes.

Kuryłowicz et al. (1987,1991a) reported a location in small vesicles. These vesicles were assigned as Golgi-like vesicles on the base of ultrastructural criteria. With the help of antibodies against ACVS, Müller et al. (1991) detected ACVS in a 100, 000-*g* pellet that contains small organelles and membrane vesicles. In these experiments, the behaviour of ACVS was notably distinct from that of IPNS and IAT, which are located in the cytosol and in microbodies, respectively (*vide infra*). On the base of marker enzyme activities, Lendenfeld et al. (1993) assigned the 100, 000-*g* pellet fraction to vacuolar vesicles. They also associated ACVS with intactly isolated vacuoles. Disruption studies with these vacuoles pointed to a loose attachment of ACVS to either the inner or the outer side of the vacuolar membrane. This finding, and indications that ACVS withdraws its substrate amino-acid precursors from a pool which is separated from the cytosolic pool and probably resides in the vacuole (*vide infra*; Section 3), led to the hypothesis that ACVS is located inside the vacuole (Lendenfeld et al., 1993).

Although Kuryłowicz et al. (1987,1991a), Müller et al. (1991), and Lendenfeld et al. (1993), have interpreted their results differently, they all started their localisation studies with *P. chrysogenum* protoplasts. These protoplasts were lysed by an osmotic shock. Lendenfeld et al. (1993) indicated that the localisation of ACVS in small vesicles by Kuryłowicz et al. (1987,1991a) and Müller et al. (1991) was due to relatively fiercer osmotic shocks applied by the latter authors compared to their own conditions. The structural criteria that led Kuryłowicz et al. (1987,1991a) to assign the vesicles as Golgi vesicles were probably not unambiguous.

Despite the fact that the mentioned observations point to a vacuolar location of ACVS, it is doubtful whether the findings reflect the active status of ACVS in Pen biosynthesis. First, protoplastation of *P. chrysogenum* is a process that takes 3–24 h under starvation conditions (Müller et al., 1991; Lendenfeld et al., 1993). This induces degradative processes which is indicated, e.g., by substantial vacuolisation (Paul et al., 1994; Paul & Thomas, 1996; Pusztahelyi et al., 1997ab; van de Kamp et al., unpublished). Second, purified ACVS has a rather sharp pH optimum above 8.0 (Baldwin et al., 1991; Theilgaard et al., 1997) (Table 1). This contrast with the vacuolar pH, which is about 1–2 pH units lower than the cytosolic pH of

approx. 7 (Sanders & Slayman, 1982; Klionsky et al., 1990; Roncal et al., 1993; Wada & Anraku, 1994; Jones et al., 1997). Third, no identifiable sequence which could target ACVS to either endoplasmatic reticulum (Gomord & Faye, 1996; Harter & Wieland, 1996; Pelham, 1996) or vacuole (Conibear & Stevens, 1995; Gomord & Faye, 1996; Van den Hazel et al., 1996; Jones et al., 1997; Klionsky, 1997,1998; Bryant & Stevens, 1998), has been recognised so far. Fourth, ACVS is extremely unstable (van Liempt et al., 1989; Zhang & Demain, 1992a; Zhang et al., 1992; Theilgaard et al., 1997), and it is not conceivable how such a vulnerable multi-domain enzyme could function in the proteolytic environment of the vacuole (Klionsky et al., 1990; van den Hazel et al., 1996; Jones et al., 1997). These points argue against a (physiologically important) location of ACVS inside vacuoles.

Additional arguments plea against a functional location wherein ACVS would be attached to or inserted in a lipid environment. First, purified ACVS behaves as a soluble (though highly unstable) enzyme in the absence of detergents, and its activity appears not dependent on the presence of detergents or lipids (Van Liempt et al., 1989; Baldwin et al., 1990,1991; Theilgaard et al., 1997). Second, sequence analysis indicates that ACVS is a hydrophobic protein, but it has not indicated the presence of specific membrane-spanning or -interacting structural elements (Lendenfeld et al., 1993). Taken together, these data do not corroborate with a functional vacuolar, or vacuolar membrane-associated location.

In line with these arguments, it was recently found that in subcellular fractionation experiments carried out with *P. chrysogenum* mycelium that had been rapidly disrupted (minute time-scale), ACVS did not co-sediment with vacuolar markers (van de Kamp et al., unpublished). Instead, it behaved as a cytosolic protein. A cytosolic location would be in accordance with the apparently unglycosylated nature of the purified ACVSes from *P. chrysogenum* and *C. acremonium* (Zhang & Demain, 1992a; Theilgaard et al., 1997). *A. nidulans* ACVS, however, was reported to be glycosylated (MacCabe et al., 1991a). The presumed prokaryotic origin of the ACVS-encoding gene (Peñalva et al., 1991; Aharonowitz et al., 1992; but see Smith et al., 1992) argues in favour of a location similar to that of bacterial ACVSes, i.e., the cytosol. Direct localisation by immunogold labelling experiments using antibodies raised against ACVS from *A. nidulans* or *P. chrysogenum* failed sofar, however, due to the absence of a specific reaction (Müller et al., 1991; Lendenfeld et al., 1993). Though final clarity is thus still lacking, we putatively conclude that ACVS is predominantly located in the cytosol (Figs. 2 and 5).

2.2 Isopenicillin N synthase (IPNS)

The enzyme IPNS (Table 1) catalyses the second step in both Pen and Ceph biosynthesis. In this step both the β-lactam and the thiazolidine rings, which together build the penam nucleus, are created (Ramos et al., 1985; Baldwin et al., 1987) (Fig. 1). Kuryłowicz et al., (1987) and Kurzątkowski et al., (1991a,b) reported that IPNS would be located in Golgi vesicles and in the cell wall, on the base of an enzymatic assay, and on the base of immunogold labelling experiments applying antibodies raised against *C. acremonium* IPNS, respectively. However, cell fractionation experiments clearly demonstrate that IPNS behaves as a soluble, cytosolic protein (Müller et al., 1991) (Figs. 2 and 5). This is in line with the absence of targeting or transmembrane sequences (Samson et al., 1985; Carr et al., 1986; Ramón et al., 1987; Barredo et al., 1989a), and with all biochemical (pH optimum 7.8) and structural data available for the enzyme (Martín & Liras 1989; Roach et al., 1995,1997).

2.3 Acyl-coenzyme A:isopenicillin N acyltransferase (IAT)

The hetero-dimeric enzyme IAT (Table 1) catalyses the third step in Pen biosynthesis. In this step an acyl group, e.g., phenylacetyl (PA) or phenoxyacetyl (POA), is substituted for the Aad side-chain of IPN (Alvarez et al., 1987,1993; Whiteman et al., 1990) (Figs. 1 and 3b). IAT is not involved in Ceph biosynthesis. Kuryłowicz et al. (1987,1991a) indicated a location of IAT in Golgi vesicles, but more recent results have demonstrated that the enzyme is located in microbodies (Figs. 2 and 5). By using antibodies against IAT, the protein was detected in a subcellular fraction that contained microbody-like organelles (Müller et al., 1991). Confinement by microbodies was shown by immunogold labelling in combination with electron microscopy performed on whole cells (Müller, 1991; Müller et al., 1991) and on subcellular fractions enriched in microbodies (Müller et al., 1995). IAT appears to be a freely soluble protein inside these organelles. The 28-kDa subunits of the IATs of *P. chrysogenum* and *A. nidulans* contain a typical C-terminal 3-amino acid peroxisomal targeting sequence (PTS1), viz. ARL and ANI, respectively (Barredo et al., 1989b; Montenegro et al., 1990; Tobin et al.,

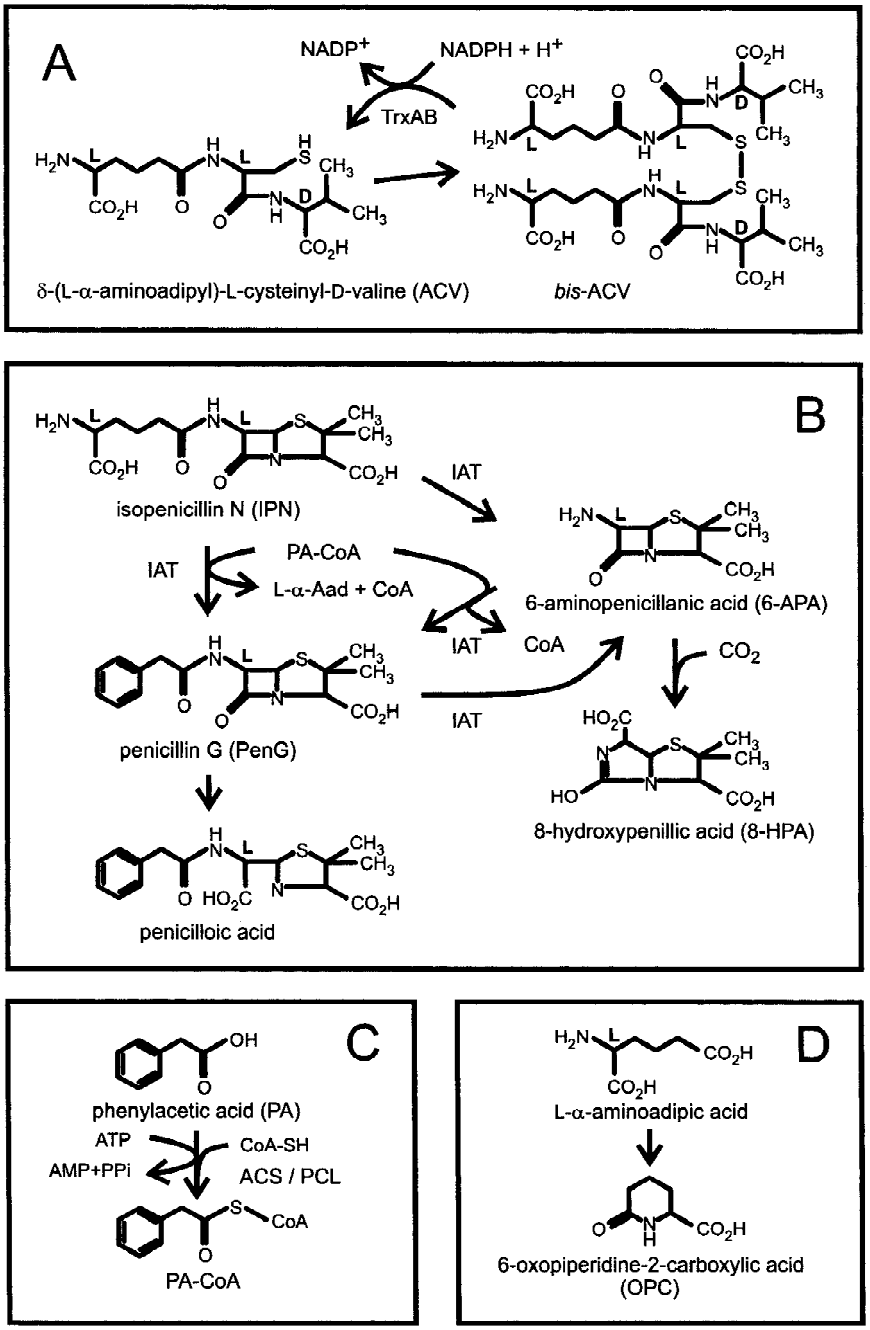

Figure 3. (A) Formation and reduction of bis-ACV. (B) Formation of the by-products of penicillin G biosynthesis, 6-aminopenicillanic acid (6-APA), 8-hydroxypenillic acid (8-HPA) and penicilloic acid. (C) Activation of the side-chain precursor phenylacetic acid (PA) in penicillin G biosynthesis. (D) Formation of the side-product 6-oxopiperidine-2-carboxylic acid (OPC).

1990). Deletion of this tripeptide from the *Penicillium* IAT leads to mis-targeting of the active enzyme to small vacuoles and their vicinity (Müller et al., 1992). Interestingly, no Pen production occurs in this situation. Also, a role for Gly150 in proper deployment of IAT has been proposed (Fernández et al., 1994).

IAT is synthesized as a full length 357-amino acid preprotein and undergoes autocatalytic processing to yield the heterodimeric protein (Table 1). Heterologous expression in *Escherichia coli* (*E. coli*) and *C. acremonium*, and *in vitro* transcription/translation of the IAT-encoding *penDE* gene from *P. chrysogenum*, resulted in active, processed IAT (Gutiérrez et al., 1991b; Aplin et al., 1993; Tobin et al., 1993). The *A. nidulans* IAT appears less capable to process itself (Fernández et al., 1998). IAT is likely to be imported into microbodies in its processed form, similar to what has been shown for other oligomeric proteins (Glover et al., 1994; McNew & Goodman, 1994; Walton et al., 1995; Subramani, 1996; Waterham & Cregg, 1996).

2.4 Cephalosporin biosynthesis-specific proteins

After the synthesis of isopenicillin N (IPN), the Pen and Ceph biosynthesis pathways diverge (Fig. 1). Ceph biosynthesis proceeds with the epimerisation of IPN by IPN epimerase (IPNE) (Fig. 1; Table 1). *P. chrysogenum* exhibits almost no, if any IPNE or IPNE-like activity (Cantwell et al., 1992; Alvi et al., 1995). Moreover, no *P. chrysogenum* genes homologous to the IPNE-encoding *cefD* and DAOCS-encoding *cefE* genes from *S. clavuligerus* have been detected (Cantwell et al., 1992). IPNE activity has been detected for *C. acremonium* but has only been poorly characterised due to the low stability of the protein (Baldwin et al., 1981; Lübbe et al., 1986). Also, the IPNE-encoding gene has not been cloned from *C. acremonium* (Martín & Gutíerrez 1995; Díez et al., 1997). However, the IPNE-encoding *cefD* genes of the cephamycin-producing actinomycetes *S. clavuligerus* and *Nocardia lactamdurans* (*N. lactamdurans*) have been cloned (Kovacevic et al., 1990; Coque et al., 1993b), and their products have been well characterised (Usui & Yu, 1989; Láiz et al., 1990) (Table 1).

PenN undergoes expansion of the thiazolidine ring to a dihydrothiazine ring which builds, together with the β-lactam ring, the characteristic cephem nucleus, yielding deacetoxycephalosporin C (DAOC) (Fig. 1). DAOC is subsequently hydroxylated at the 3′-cephem position to give deacetylcephalosporin C (DAC) (Fig. 1). In *C. acremonium*, DAOC synthesis and hydroxylation (or DAC synthesis) are catalysed by the bi-functional DAOCS/DACS enzyme encoded by the mono-cistronic *cefEF* gene, which are both well characterised (Dotzlaf & Yeh, 1987; Samson et al., 1987; Yeh et al., 1991) (Table 1). Finally, DAC is acetylated by the *cefG*-gene encoded enzyme acetyl-CoA:DAC acetyltransferase (Fujisawa & Kanzaki, 1975; Liersch et al., 1976; Félix et al., 1980; Gutiérrez et al., 1992; Matsuda et al., 1992; Mathison et al., 1993) (Table 1), yielding Ceph C (Fig. 1). IPNE, DAOCS/DACS, and DAT behave as soluble cytosolic proteins with pH optima above 7.0 (Table 1). No indications are presently available for a compartmentalization of any of these enzymes (Table 1; Fig. 2).

2.5 Auxiliary proteins

Apart from the dedicated proteins involved in Pen and Ceph biosynthesis, a number of enzymes involved in primary metabolism may interfere or are prerequisite for β-lactam antibiotic formation. Some of them will be discussed here.

Acetyl-CoA synthetase (ACS) and phenylacetyl-CoA ligase (PCL).

In Pen biosynthesis, the aminoadipyl side-chain of IPN is commonly exchanged with either a PA or a POA side-chain, yielding PenG and PenV, respectively, by the microbody-located IAT. Prior to this reaction, PA and POA have to be activated to their CoA thioesters by an enzyme with acetyl-CoA synthetase activity (Fig. 3c). A gene encoding a cytosolic acetyl-CoA synthetase (ACS) (Figs. 2 and 5) has been isolated from *A. nidulans* and *P. chrysogenum* (*acuA=facA*) (Connerton et al., 1990; Gouka et al., 1993; Martínez-Blanco et al., 1993). ACS has been characterised (Table 1), and could be used to produce Pens in an *in vitro* system (Martínez-Blanco et al., 1992). Putatively different ACS-like enzymes have been described as well (Brunner & Röhr, 1975; Kogekar & Deshpande, 1982). Recently, such a PA-activating ACS-like enzyme, called PA-CoA ligase (PCL), was isolated and its encoding gene cloned (International patent WO97/02349) (Table 1). Different from the cytosolic ACS mentioned above, PCL has a PTS1 sequence (SKI). This indicates its targeting to the microbody where it could function in conjunction with IAT (Figs 2 and 5). *In vitro*, PCL functions best around its pH optimum, 9.0 (International patent WO97/02349), whereas IAT has a rather

narrow pH working range around its pH optimum, 8.0 (Alvarez et al., 1987,1993) (Table 1). This implores an alkaline environment for the last reaction(s) in Pen biosynthesis (*vide infra*, Section 4.3).

It is unclear whether either one or both of the mentioned enzymes ACS and PCL are involved in Pen biosynthesis. PA activation by ACS appears poor (Martínez-Blanco et al., 1992), and disruption of the *acuA* gene does not affect Pen biosynthesis (International patent WO92/07079; Gouka et al., 1993). Overproduction of PCL, on the other hand, does not appear to result in a higher Pen production, either (International patent WO97/02349). Interestingly, overproduction in *P. chrysogenum* of the *pcl* gene from *Pseudomonas putida* U, which encodes a PA-CoA ligase which most probably resides in the fungal cytosol (Table 1), increased Pen production two-fold (Miñambres et al., 1996), indicating a possible role for a cytosolic enzyme. An alternative P(O)A-activating route involving glutathione *S*-derivation has been proposed which involves the action of cytosolic GSH S-transferase (GST; *vide infra*) (Ferrero et al., 1990). The corresponding GSH S-conjugate of PA, GSPA (Fig. 4), is a good substrate for IAT (Alvarez et al., 1993). However, no evidence has been provided for either the functioning or the significance of this route *in vivo*.

Disulphide reductases

Once ACV is synthesized in the cell, it is capable of dimerising to *bis*-ACV (Fig. 3a), which withdraws it from further processing by IPNS. Moreover, *bis*-ACV inhibits the activity of ACVS (Theilgaard et al., 1997). Re-entering of *bis*-ACV in Pen biosynthesis is possible by the action of a NADPH-dependent thioredoxin-based broad-range disulphide reductase system (TrxAB) which has been characterised in *P. chrysogenum* at the protein and gene level (Cohen et al., 1994) (Figs 3a and 4; Table 1). A similar system is encountered in cephamycin-producing *S. clavuligerus* (Aharonowitz et al., 1993b) (Table 1). The TrxAB reductase system probably functions in the cytosol (Fig. 5). As an alternative, *bis*-ACV can also be reduced by a GSH disulphide reductase (GSHred; Table 1) (Cohen et al., 1994) (Fig. 4). ACV may also form mixed disulphides with other thiol-containing compounds, e.g., with GSH (vide infra). These might also be reduced by either of these reductases.

β-Lactam antibiotic acylases

Some reports have appeared concerned with the presence of a Pen acylase in *P. chrysogenum* (Erickson & Bennett, 1965; Vanderhaeghe et al., 1968; Meesschaert et al., 1991; Alvarez et al., 1993; Gil-Espinosa et al., 1993). Acylase activity may be partly responsible for the formation of 6-aminopenicillanic acid (6-APA) during fermentations (Fig. 3b). The Pen acylase has a putative cytosolic location (Fig. 5).

Catalase

Catalase-activity might be involved in the formation of *p*- or *o*-hydroxy-Pens, via hydroxylation of the (CoA-thioesterified) side-chain precursors (Erdélyi et al., 1966; Adlard et al., 1991; Christensen et al., 1994a; Nielsen 1995). In *A. nidulans* and *P. chrysogenum*, catalase is located inside microbodies (Müller et al., 1991; Valenciano et al., 1996,1998).

Enzymes involved in glutathione metabolism

Glutathione [γ(-L-glutamyl)-L-cysteinyl-glycine; GSH] (Meister & Anderson, 1983; Meister, 1988; Penninckx & Elskens, 1993; Noctor & Foyer, 1998) is a close analog of the ACV tripeptide (Fig. 4). This is illustrated by its capability to inhibit IPNS (Ramos et al., 1985). GSH can potentially be converted to the Pen-like glutacillin which closely resembles IPN (Spallholz, 1987). GSH may serve diverse roles, e.g., in the storage and supply of the constituing amino acids, in the regulation of S-metabolism, in the protection of the cell against potentially harmful compounds, in the regulation of the oxidative state of the cytosol, in the sensing of extracellular amino acid availability, and in the transport of amino acids (Meister & Anderson, 1983; Meister, 1988; Penninckx & Elskens, 1993; Noctor & Foyer, 1998). GSH metabolism largely proceeds via the γ-glutamyl-cycle (Meister & Anderson, 1983; Meister, 1988) which involves the enzymes γ-glutamylcysteine synthetase, GSH synthetase, γ-glutamyltranspeptidase (γGT), γ-glutamylcyclotransferase (γGCT), and 5-oxoprolinase (5OP) (Fig. 4). The enzymes of the γ-glutamyl cycle are located in the cytosol, with the exception of γGT (Fig. 4). This enzyme, which may be involved in the uptake or mobilisation of amino acids (Meister & Anderson, 1983; Meister, 1988; Penninckx & Elskens, 1993; Payne & Smith, 1994; Mehdi & Penninckx, 1997), has been reported to reside in the cell wall (Payne & Payne, 1984; del Carmen Mateos & Sánchez, 1990) and in the vacuole (Jaspers & Penninckx, 1984). Oxidised GSH, GSSG, and mixed sul-

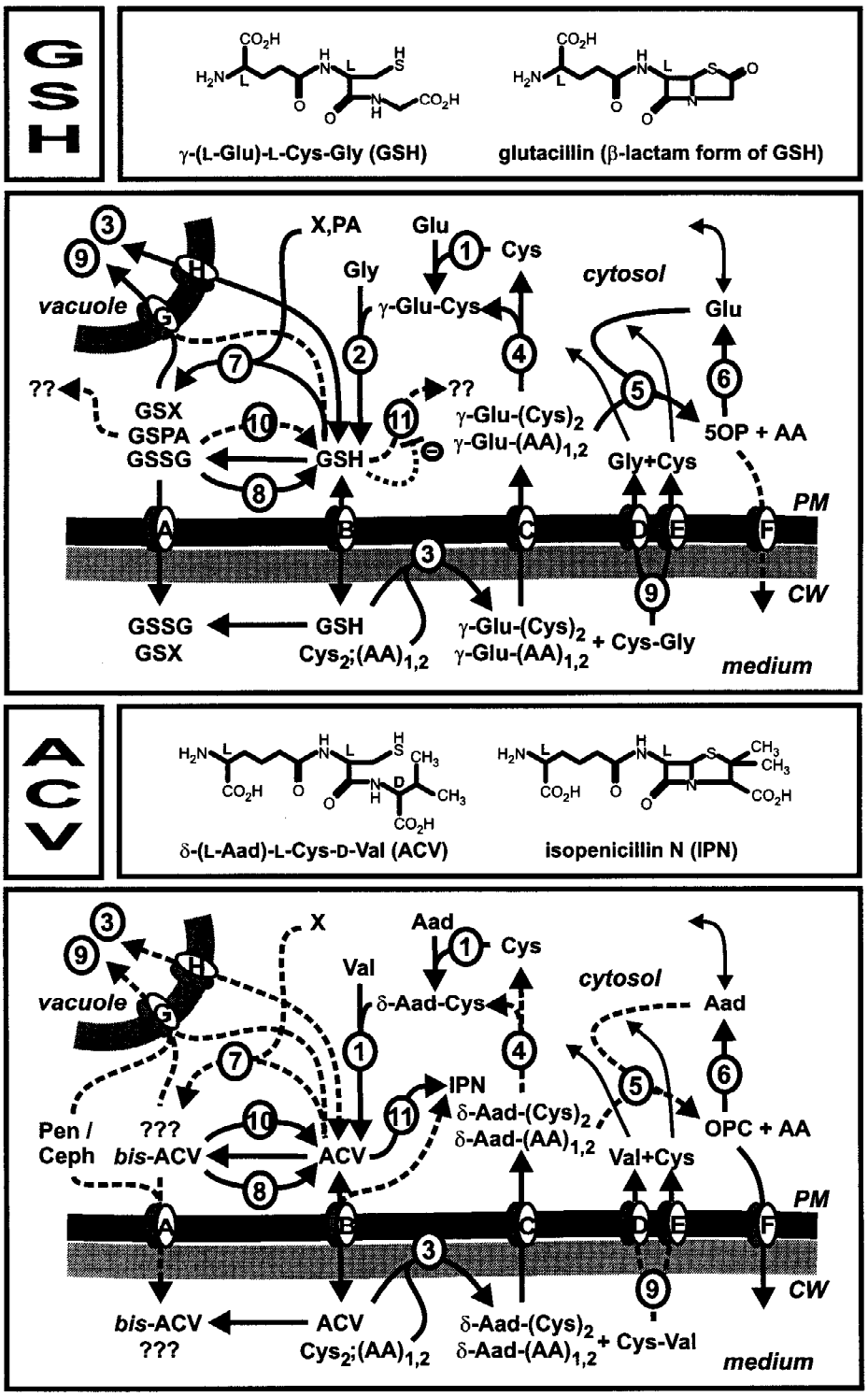

Figure 4. Resemblance of glutathione (GSH) and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) structures and metabolism. Enzymes: (1) γ-glutamylcysteine synthetase (top panel) or ACV synthetase (bottom panel); (2) GSH synthetase; (3) γGT; (4) GSH thiol transferase; (5) γGCT; (6) 5OP; (7) GST; (8) GSHred; (9) (di)peptidase(s); (10) TrxAB; (11) IPNS. Transport steps: A and G, GSX transporters; B and H, GSH transporter; C, γ-glutamyl-amino-acid uptake system; D and E, amino-acid uptake systems; F, OPC transport system. Putative enzymatic steps are indicated by dashed arrows. PM, plasma membrane; CW, cell wall.

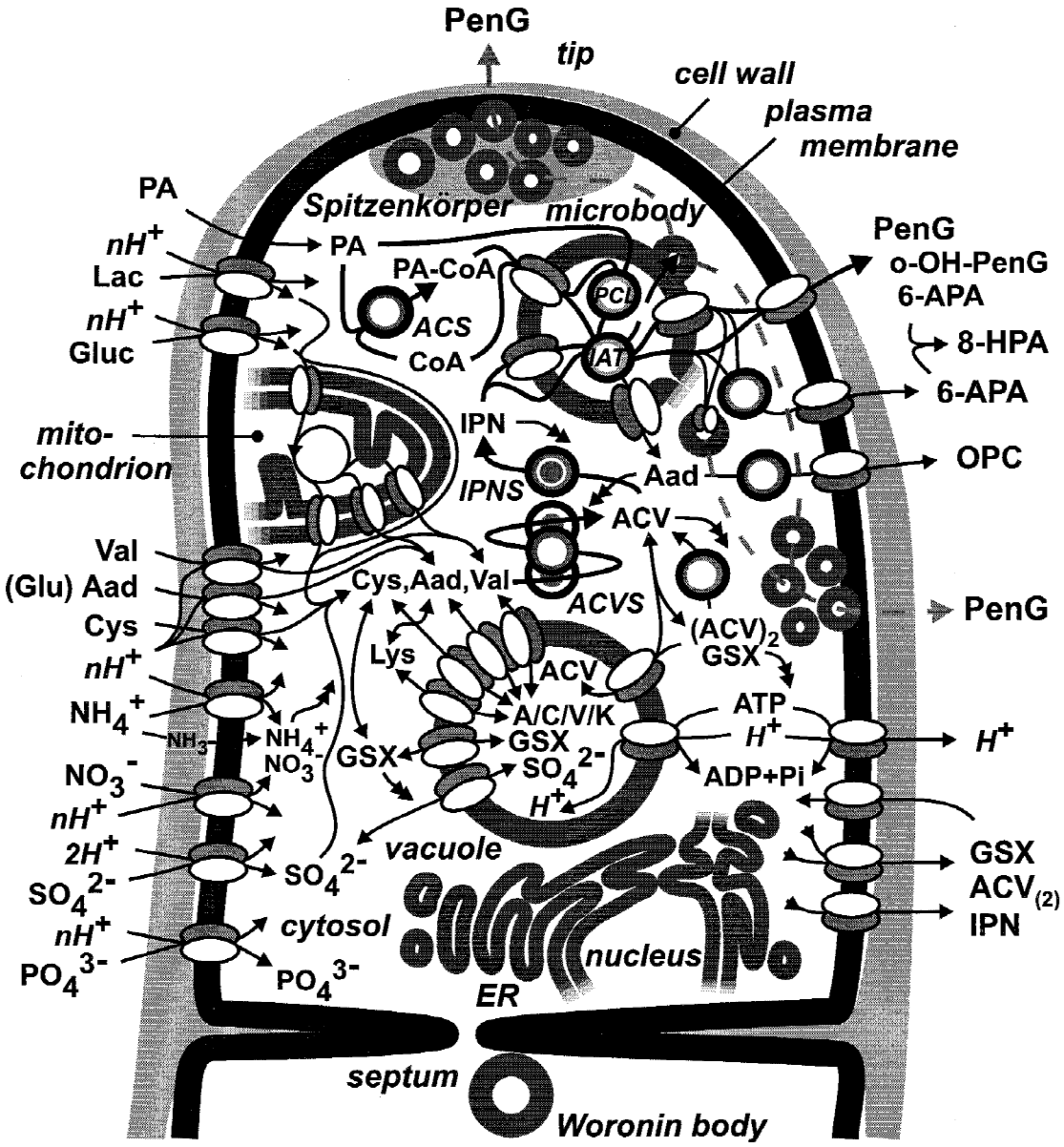

Figure 5. Model depicting compartmentalization and selected (putative) transport steps involved in the biosynthesis of penicillin G by *Penicillium chrysogenum.* Enzymes and transporters are discussed in the text. Some putative enzymatic and transport steps are depicted as parallel routes.

phides of GSH with thiol-containing compounds (e.g., ACV) can be reduced by GSHred which is putatively located in the cytosol (Fig. 4). Potentially toxic compounds may form S-conjugates with cytosolic GSH by the action of GSH *S*-transferase (GST). These conjugates are transported into the vacuole or expelled via transporters in the plasma membrane (*vide infra*, Section 4) (Fig. 4). It is unsure whether all the mentioned enzymes that are involved in GSH metabolism, are present in fungi (Penninckx & Elskens, 1993; Henriksen, 1996).

Because GSH resembles ACV (as well as other Pen and Ceph biosynthesis intermediates and products), it is tempting to speculate that GSH and ACV metabolism have several elements in common or interfere with each other (Fig. 4). ACV might be a substrate

or an inhibitor of GSH- or GSX-processing and transporting enzymes (GSX stands for GSH-conjugate). It has been repeatedly shown or suggested that Pen biosynthesis is interconnected with GSH metabolism (e.g. Sanchez et al., 1988; Schwartz et al., 1988; Ferrero et al., 1990; del Carmen Mateos & Sanchez, 1990; Nielsen, 1995; Henriksen, 1996; Emri et al., 1997,1998; Brakhage, 1998; Henriksen et al., 1998). For example, GST, γGT and GSHred are induced, the GSH pool is depleted, and the oxidised GSH concentration has been found to be increased, when *P. chrysogenum* is grown under Pen-producing conditions in the presence of glutamate as N-source, or when lactose or carbon-starvation conditions were used instead of growth on glucose (Emri et al., 1997,1998). Further examples are spread throughout this review.

3. Compartmentalization of precursors, intermediates and products

For a full understanding of the physiology of β-lactam production, not only the location of enzymes, but also the distribution of precursors, intermediates and products, should be considered (Jørgensen et al., 1995a). Because Pen biosynthesis is partly compartmentalized but Ceph biosynthesis is not, and because data about intracellular concentrations of Ceph and Ceph biosynthesis intermediates and side-products are not available to the extent they are for Pen and Pen biosynthesis intermediates, the main focus here will be on Pen biosynthesis. Where appropriate, attention will be given to Ceph biosynthesis as well.

3.1 Precursor amino acids

Pen and Ceph are synthesized starting from α-aminoadipate (Aad), cysteine (Cys), and valine (Val) (Fig. 1). Of these amino acids, Aad appears limiting for Pen biosynthesis. A positive correlation exists between its internal concentration and Pen production (Friedrich & Demain, 1978; Jaklitsch et al., 1986; Revilla et al., 1986; Hönlinger & Kubicek, 1989b), which levels off at higher concentrations (Jørgensen et al., 1995b). Aad is an intermediate of lysine biosynthesis in fungi (Bhattacharjee, 1985,1992; Kubicek-Pranz & Kubicek, 1991). Its concentration is dependent on the equilibrium between lysine anabolism and catabolism (Hönlinger & Kubicek, 1989a; Lu et al., 1992; Esmahan et al., 1994) and can be increased by feeding Aad (Friedrich & Demain, 1978; Jaklitsch et al., 1986). The flux from Aad to either Lys or Pen can be manipulated by genetic engineering of the lysine biosynthesis pathway (Gutiérrez et al., 1998). Cys can be synthesized by three different pathways, called the direct sulphydrylation, the transsulfuration and the reverse transsulfuration pathways (Kubicek-Pranz & Kubicek, 1991; Marzluf, 1997a; Thomas & Surdin-Kerjan, 1997; Sieńko et al., 1998). In *A. nidulans* direct sulphydrylation appears the main route; for *P. chrysogenum* and *C. acremonium*, the transsulfuration pathway has been suggested to be primarily used (Nüesch et al., 1987; Marzluf, 1997a; Sieńko et al., 1998). Use of the reverse transsulfuration pathway by *C. acremonium*, in which methionine (Met) is an intermediate, may explain why addition of Met to the growth medium results in an elevated Ceph production (Velasco et al., 1994). Valine biosynthesis is closely related to the biosynthesis of leucine and isoleucine (Nüesch et al., 1987; Kubicek-Pranz & Kubicek, 1991). Of all these amino acid biosynthetic routes, significant parts are compartmentalized in mitochondria (Kubicek-Pranz & Kubicek, 1991; Marzluf, 1997a) (Fig. 5).

In *P. chrysogenum*, Aad is, together with Val and Lys and possibly Cys, sequestered from the cytosol into a vacuolar pool (Hönlinger and Kubicek, 1989ab; Kubicek et al., 1990; Affenzeller et al., 1991; Lendenfeld et al., 1993). Total intracellular concentrations of the amino acids Aad, Cys, Val and Lys are approx. $<$0.1–1.4, 0.1–0.4, 1.1–2.4, and 1.1–1.5 mM (dependent on strain and conditions), and more than 90% of the Lys pool may reside in the vacuole (Jaklitsch et al., 1986; Hönlinger & Kubicek, 1989ab; Kubicek et al., 1990; Kubicek-Pranz & Kubicek, 1991; Jørgensen, 1993; Jørgensen et al., 1995b; Henriksen et al., 1996) [for conversion of mg/g dry weight (dw) values to concentrations, a hyphal density value of 2.5 ml/g dw was used (Packer et al., 1992; Jørgensen et al., 1995b)]. The intracellular amino acid concentrations are important in view of the affinity of ACVS for its substrates. *P. chrysogenum* ACVS has affinities in the sub-mM range (Table 1) (Theilgaard et al., 1997); so, the intracellular amino acid concentrations indicate saturation of ACVS with its amino acid substrates. It is noted that the ACVSes from *C. acremonium* and *S. clavuligerus* have significantly lower affinities (Schwecke et al., 1992; Zhang et al., 1992a; Kadima et al., 1995; Kallow et al., 1998) (Table 1). It has been indicated that the amino acids that are incorporated in ACV are withdrawn specifically from the vacuolar pool (Hönlinger & Kubicek, 1989a;

Affenzeller & Kubicek, 1991). Exchange between cytosolic and vacuolar pools may be an important step in controlling Pen biosynthesis (Affenzeller & Kubicek, 1991; Lendenfeld et al., 1993). Also, differences in distribution between vacuolar and cytosolic pools may contribute to *P. chrysogenum* productivity (Hönlinger & Kubicek, 1989a; Affenzeller & Kubicek, 1991). Evidence exists that sulphate, which is essential for Cys biosynthesis, is sequestered in the vacuole as well (Hunter & Segel, 1985). Finally, glutamate (Glu), which is the most abundant intracellular amino acid (10–40 mm; Jaklitsch et al., 1986; Jørgensen, 1993; Henriksen et al., 1996) is worth considering, e.g., for its central role in N-metabolism and for the relationship between the Glu pool and antibiotic biosynthesis (Jaklitsch et al., 1985; Lara et al., 1982). Dehydrogenation of Glu yields α-ketoglutarate (αKG), which is a precursor for Aad biosynthesis; moreover, αKG is a cosubstrate for the expandase/hydroxylase reactions in Ceph biosynthesis (Dotzlaf & Yeh, 1987; Yeh et al., 1991). Glu is a predominantly cytosolic amino acid (Kubicek-Pranz & Kubicek, 1991).

*3.2 δ-(*L*-α-Aminoadipyl)-*L*-cysteinyl-*D*-valine (ACV), glutathione (GSH), isopenicillin N (IPN), 6-oxopiperidine-2-carboxylic acid (OPC), and cephalosporin biosynthesis intermediates*

The total intracellular ACV content during high-level Pen production by *P. chrysogenum* increases from 0.25 to 4.0–6.5 mm (Jørgensen, 1993; Jørgensen et al., 1995b; Nielsen, 1995). According to metabolic control analysis, this increase would correlate with a shift in rate-limitation from ACVS to IPNS; IAT was not considered to be rate-limiting at any stage (Nielsen & Jørgensen, 1995; Nielsen, 1998). This analysis is consistent with increased Pen production by *A. nidulans* in which the ACVS-encoding *acvA* (=*pcbAB*) gene is overexpressed (MacCabe et al., 1991b; Kennedy & Turner, 1996) and with unaffected Pen production by *A. nidulans* in which the IAT-encoding *acyA* (=*penDE*) gene is overexpressed (Fernández-Cañón & Peñalva, 1995b). Perhaps unexpectedly, overexpression of the IPNS-encoding *ipnA* gene in *A. nidulans* did not result in elevated Pen production (Fernández-Cañón & Peñalva, 1995b). In contrast to these data obtained for *A. nidulans*, overexpression studies with *P. chrysogenum* are not fully corroborative with the results from metabolic control analysis (Veenstra et al., 1991; van den Berg et al., 1998).

A high internal ACV concentration may cause inhibition of ACVS (Theilgaard et al., 1997). Part of the ACV will be present as *bis*-ACV or as mixed disulphides, which can be reduced by broad-range disulphide reductases (Cohen et al., 1994) (*vide supra*, Section 2). *Bis*-ACV inhibits ACVS (K_i 1.4 mm; Theilgaard et al., 1997). The affinity of IPNS for ACV is in the sub-mM range (Pang et al., 1984; Ramos et al., 1985; Baldwin et al., 1987) (Table 1). It has been suggested that ACV is present in the vacuole (Hönlinger & Kubicek, 1989a; Lendenfeld et al., 1993), either because it is produced inside the vacuole (but see Section *2.1*) or because it is stored in the vacuole. The ACV-analogue GSH is present in a high-producing *P. chrysogenum* strain at an intracellular concentration of 3-6 mm (Nielsen, 1995; Nielsen & Jørgensen, 1995). This is just below its K_i for IPNS (8.9 mm; Ramos et al., 1985), but high enough to serve a role in the rescuing or scavenging of ACV by the formation of mixed disulphides. GSH also inhibits ACVS (Sanchez et al., 1988). In *Saccharomyces cerevisiae* (*S. cerevisiae*), GSH can be stored in the vacuole to high levels (90%), to supply the cell with the constituent amino acids (Elskens et al., 1991; Mehdi & Penninckx, 1997). During high-level Pen production, ACV accumulates in the medium to concentrations of up to 2 mm (Jørgensen, 1993; Jørgensen et al., 1995b; Nielsen, 1995). GSH is present in the medium at concentrations up to 0.3 mm (Jørgensen, 1993).

The intracellular IPN concentration during high-level production by *P. chrysogenum* is in the range of 0.5–1.0 mm (Jørgensen, 1993; Jørgensen et al., 1995b; Nielsen, 1995). IPN does not accumulate in the medium; its extracellular concentration remains approx. constant at 0.2–0.4 mm (Jørgensen, 1993; Nielsen, 1995). The affinity of the microbody-located IAT from *P. chrysogenum* for IPN lies in the μM-range (Alvarez et al., 1993) (Table 1). No data are available on the compartmentalization of IPN.

OPC (6-oxopiperidine-2-carboxylic acid), a circular isomer of Aad (del Carmen Mateos & Sanchez, 1990; Henriksen, 1996; Henriksen et al., 1998) (Fig. 3d), is found extracellularly (Fig. 5) at a concentration that keeps in pace with Pen production. It is not detected intracellularly (Hönlinger & Kubicek, 1989b). The ratio between OPC and Pen production is dependent on strain and growth medium (Brundidge et al., 1980; Herschbach et al., 1984; Revilla et al., 1986; Jørgensen et al., 1995b; Henriksen, 1996; Henriksen et al., 1998). OPC formation and excretion may be a mechanism for the cell to deal with high intracellular

concentrations of Aad, which in itself may be toxic (Hönlinger & Kubicek, 1989b). OPC formation may be directly linked to IAT activity. However, it is more likely due to the action of an enzyme which is not involved in Pen biosynthesis, such as γGCT, for example (Henriksen, 1996; Henriksen et al., 1998) (Fig. 4). OPC can be decyclized to Aad by 5OP (van der Werf et al., 1975; Griffith & Meister, 1981) (Fig. 4). OPC can be used as a substrate, instead of Aad, for ACVS from *N. lactamdurans* (Coque et al., 1996a).

Data for Ceph biosynthesis intermediates are scarce. A rate-limiting bottle-neck has been found at the conversion of DAC by DAT. DAC accumulates extracellularly together with PenN during Ceph biosynthesis (Fujisawa et al., 1973; Nüesch et al., 1987; Skatrud et al., 1989). Increasing the gene-dosage or overexpression of the DAT-encoding *cefG* gene, either or not concomittantly with the expandase/hydroxylase-encoding *cefEF* gene, resulted in increased Ceph production and much decreased PenN and DAC levels (Skatrud et al., 1989; Mathison et al., 1993; Gutiérrez et al., 1997).

3.3 Side-chain precursors phenylacetic acid (PA) and phenoxyacetic acid (POA)

PA and POA (pK_a values of 4.3 and 3.1, respectively) will tend to be distributed across the plasma membrane according to the pH gradient (ΔpH, alkaline inside). Depending on the external concentration, this may lead to intracellular accumulation to up to hundreds of mM (Hunter & Segel, 1973b; Eriksen et al., 1995; Hillenga et al., 1995; Henriksen, 1996). The affinity of ACS and PCL for P(O)A is not known; both high (μM range) and low (mM range) affinities have been suggested (Martínez-Blanco et al., 1992; Henriksen, 1996). PA is preferred over POA when supplied at similar extracellular concentrations (Eriksen et al., 1994). This has been related to the putative affinities of the activating enzyme for PA and POA (Henriksen 1996). The affinity of the *P. chrysogenum* IAT for PA-CoA is 6.0 μM (Alvarez et al., 1993) (Table 1); the affinity for POA-CoA is slightly lower (Alvarez et al., 1993). It has been indicated that the extracellular concentration of POA must be kept high ($>$30 mm) in order to prevent substantial formation of 6-APA and 8-hydroxypenillic acid (8-HPA) as by-products, and to prevent loss of IPN to the medium. However, a high POA concentration increases the excreted levels of ACV and OPC (Henriksen, 1996).

PA can be hydroxylated to *o*-OH-PA (and POA to *p*-OH-PA). The loss of PA by metabolism in *P. chrysogenum* has been reported to be low (Hillenga et al., 1995). Nevertheless, hydroxylated compounds are found as a significant fraction in the broth during industrial fermentations (Adlard et al., 1991); *o*- and *p*-OH-Pens may make up 20% of the final yield (Christensen et al., 1994a; Nielsen, 1995). *o*-OH-PA can be metabolised further to enter the phenylalanine degradation pathway as homogentisate (Fernández-Cañón & Peñalva, 1995a). The hydroxylated side-chain precursors can be used as substrates for the IAT, yielding *o*- and *p*-OH-Pens, but the affinity of IAT for these substrates is lower than for the unhydroxylated P(O)A (Alonso et al., 1988). Disruption of the PA-hydroxylase encoding gene improved the efficiency of Pen production in *A. nidulans* (Rodriguez et al., 1998). It has been suggested that *o*- and *p*-OH-Pens alternatively are the product of microbody-located catalase acting on (CoA-thioesterified) P(O)A (Erdélyi et al., 1966; Nielsen, 1995). Intracellular concentrations of hydroxylated side-chain precursors, their CoA-thioesters, and the derived products, are not known.

3.4 Penicillin, cephalosporin, and penicillin biosynthesis by-products

The intracellular Pen and Ceph concentrations are unknown, but they are generally assumed to be low (1–10 mm) relative to the concentration outside [estimated to be $>$100–150 mm for high-production strains (Swartz, 1985; Smith, 1985; Crueger & Crueger, 1990; Sohn et al., 1994; Nielsen, 1995; Newbert et al., 1997; Martín et al., 1997)]. In the case of Pen biosynthesis by *P. chrysogenum*, the intracellular Pen concentration could thus be equal to or just higher than the concentration of, e.g., ACV and IPN. If Pen would be distributed across the plasma membrane according to the ΔpH, its internal concentration would be an order of magnitude higher than the concentration in the medium. However, this is unlikely in view of Pen's putative toxicity due to lipophilic interactions with plasma and intracellular membranes (Sikkema et al., 1995), and in view of the applied random selection for strains with intrinsically high excretion capability (Newbert et al., 1997). Moreover, Pen is a substrate for IAT [K_m 2.0 mm (Alvarez et al., 1993)] (Fig. 3b) and for another enzyme with Pen amidase activity, called Pen acylase (Alvarez et al., 1993; Gil-Espinosa et al., 1993). High intracellular Pen concentrations would

therefore lead either to the production of high levels of 6-APA, or to futile cycling. However, no indications exist for either of these events, pointing to efficient expelling of Pen from IAT- and/or acylase-containing compartments.

Ultrastructural analysis led Kurzątkowski et al., (1987,1991) to indicate the presence of Pen inside vesicles close to the plasma membrane, but this localization is ambiguous. Suggested pre-accumulation of Pen in bulges, which would release the antibiotic by bursting (Luengo et al., 1986), has been seriously questioned by Müller (1991). No further data are available on possible intracellular distributions of Pen.

6-APA is produced as a minor side-product, and found in the broth of high-producing *P. chrysogenum* at concentrations of 0.1–0.3 mM (Jørgensen et al., 1995b; Henriksen, 1996). 6-APA formation may be the result of IAT or of Pen acylase activity (*vide supra*) (Fig. 3b), and can take place when the supply of side-chain precursors is insufficient. Note, however, that the K_m of IAT for IPN drops to 4.0 mM in the absence of P(O)A (Alvarez et al., 1993) (Table 1). 6-APA is carboxylated extracellularly to 8-HPA (Figs. 3b and 5) which accumulates to concentrations of up to 2.5 mm in the medium of high-producing *P. chrysogenum* (Jørgensen et al., 1995b; Nielsen, 1995; Henriksen et al., 1997). Penicilloic acid is detected extracellularly as the result of Pen degradation (Figs. 3b and 5), and may amount to 25% of the final Pen yield (Christensen et al., 1994a,b; Nielsen, 1995).

4. Transport of precursors, intermediates and products of β-lactam antibiotic biosynthesis

As it appears, several organelles are involved in β-lactam antibiotic biosynthesis. This invokes transport steps across intracellular boundary membranes. In addition, C-, N-, P- and S-sources, as well as some amino acid and side-change precursors have to be imported across the plasma membrane, and the produced antibiotics have to be excreted. Intracellular compartmentalization, the uptake of substrates and the release of products are often more or less ignored when metabolic fluxes are evaluated. It is worthwhile to recognize that many transport processes affect the overall energy status of the cell and are of major importance in cellular homeostasis. For this reason, transmembrane fluxes are tightly controlled and potentially determine the overall rate or levels of intermediates in various metabolic pathways (Krämer, 1996). Our current understanding of transport steps involved in β-lactam antibiotic biosynthess, though far from complete, will be considered here into some detail. An overall model depicting the cell biology of penicillin biosynthesis in *P. chrysogenum* including a selected number of purported transport steps, is given in Fig. 5.

4.1 Transport across the plasma membrane

The fungal cell wall (Kuhn et al., 1989; Ruiz-Herrera, 1992; Gooday, 1995a; Sentandreu et al., 1994; Sietsma & Wessels, 1994; van der Rest et al., 1995) is composed of a layer of polymers surrounding the plasma membrane. The cell wall can be freely traversed by solutes smaller than 700 Da, but the actual cut-off for the size of molecules that can pass the membrane may be much larger (de Nobel, 1991; de Nobel & Barnett, 1991). The plasma membrane forms the major permeability barrier between the medium and the cytosol (Kuhn et al., 1989; Gooday, 1995b; van der Rest et al., 1995). The major lipid components of the fungal plasma membrane are glycerophospholipids, combined with a characteristically high amount of sterols which tighten the lipid packing, and together with smaller amounts of sphingolipids and glycolipids (Slayman, 1987; van den Bossche, 1989; Hillenga et al., 1994; Gooday, 1995b; van der Rest et al., 1995). Generally, the content of sterols in intracellular (i.e., organellar) membranes is much lower (Lösel, 1989; Zinser & Daum, 1995).

Transport across the fungal plasma membrane can occur either by passive diffusion (mainly for relatively hydrophobic compounds), or by carrier-mediated transport. Transport proteins in filamentous fungi may be distributed inhomogeneously along the hyphae, according to the metabolic and developmental status of the mycelium (Isaac et al., 1986; Takeuchi et al., 1988; Burgstaller, 1997). Driving forces for transport in fungi are either electrochemical gradients jointly denoted as the *cmf* or Δp [cationic motive force, consisting of *pmf* or Δp_H+ (proton motive force) and *smf* or $\Delta p_{Na}+$ (sodium motive force)] (secondary transport) or ATP hydrolysis (primary transport) (van der Rest et al., 1995; Krämer, 1996). An important contributor to the *pmf* (Δp_H+; negative, alkaline inside) across the plasma membrane is the P-type (E_1E_2-type) ATPase which pumps protons out of the cell at the expense of ATP (Slayman, 1987; Stokes, 1991; Hillenga et al., 1994; André, 1995; Garril, 1995; van der Rest et al., 1995; Scarborough, 1996; Rao & Slayman, 1996; Reoyo et al., 1998)

(Fig. 5). ATP, which is used to drive ATP-dependent transport, is synthesized in the mitochondrion by the F-type (F_oF_1-type) H^+-ATPase that is present in the inner membrane (Nelson, 1992; Hillenga et al., 1994; Garril 1995). The intracellular pH is maintained at approx. 7.0–7.2 (Sanders & Slayman, 1982; Henriksen, 1996). For *A. nidulans*, grown over a wide pH range (3.5–8.0), bulk internal pH values ranging from 5.7 to 6.6 were measured, illustrating the relative robustness of the internal pH (Caddick et al., 1986). Interestingly, Pen biosynthesis is transcriptionally regulated by the external pH (Espeso et al., 1993; Tilburn et al., 1995; Suárez & Peñalva, 1996; Brakhage, 1997,1998; Chu et al., 1997).

Following this, the transport of various solutes which have a relation to β-lactam antibiotic biosynthesis, will be considered. For recent, wider reviews of plasma membrane transport in (filamentous) fungi, the following references may be consulted: Jennings (1995), van der Rest et al., (1995), Krämer (1996), Horák (1997), Burgstaller (1997), and Paulsen et al., (1998).

Sugars

β-Lactam production is optimal under conditions of nutrient imbalance and at low growth rates. This is amongst others reflected in the preferred C- and N-sources used in industrial fermentations. During fermentation of *P. chrysogenum*, a limiting glucose-feed is regularly used as C- and energy-source (Demain, 1984; Herschbach et al., 1984; Swartz, 1985). High glucose concentrations exert a negative effect on Ceph and Pen biosynthesis (Martín & Aharonowitz, 1983; Revilla et al., 1986; Nüesch et al., 1987; Demain, 1991; Brakhage et al., 1992; Espeso et al., 1992; Zhang & Demain, 1992b; Feng et al., 1994; Weil et al., 1995; Brakhage, 1997,1998). The energetically less-preferred lactose, which is frequently used in batch growth, derepresses Pen biosynthesis (Martín & Aharonowitz, 1983; Demain, 1984; Espeso et al., 1992; Brakhage, 1997,1998).

Glucose uptake systems have been described for *A. nidulans* (Mark & Romano, 1971), *A. niger* (Torres et al., 1996), and *Neurospora crassa* (*N. crassa*) (Scarborough, 1970ab; Schneider & Wiley, 1971ab; Slayman & Slayman, 1974; Madi et al., 1997). In general, two systems appear to be present, viz. a constitutive, passive, low-affinity system, and an inducible, energy-dependent, putatively H^+-symport, high-affinity system, with K_m values in the mM and the μM range, respectively. In *S. cerevisiae*, many glucose uptake systems are present, some of which have been characterised in detail and most of which exemplify facilitated diffusion systems, belonging to the sugar porter sub-family of the major facilitator superfamily (MFS) of secondary transporters (Marger & Saier, 1993; Kruckeberg, 1996; Horák, 1997; Nelissen et al., 1997; Pao et al., 1998; Paulsen et al., 1998; Saier, 1998).

Fungal lactose uptake systems are present in, e.g., *A. nidulans* (Gajewski et al., 1972; Fantes & Roberts, 1973) and *N. crassa* (Bates et al., 1967; Schneider & Wiley, 1971b). The best characterised fungal uptake systems are those of *Kluyveromyce*s species (Dickson & Barr, 1983; van den Broek et al., 1987; Chang & Dickson, 1988) which transport lactose in symport with H^+. In general, fungal disaccharide transport occurs as H^+-symport (van der Rest et al., 1995).

In *P. chrysogenum*, glucose uptake by glucose-limited or -starved mycelium appears an energy-dependent process. It is characterised by an apparent K_m of approx. 0.03 mM (Christensen et al., 1995; Nielsen, 1995; van de Kamp et al., unpublished). Lactose is taken up by *P. chrysogenum* by an energy-dependent system, which is induced by growth on lactose (van de Kamp et al., unpublished) (Fig. 5).

Ions: phosphate, ammonium, nitrate, and sulphate

The uptake of phosphate (used as P-source), ammonium, nitrate and urea (which are used as N-sources in β-lactam production) (Herschbach, 1984; Demain, 1991) has hardly been investigated in β-lactam producing fungi. High phosphate concentrations, i.e. concentrations that match the P-need of the organism, show an inhibitory effect on Ceph biosynthesis (Zhang et al., 1988; Demain, 1991). Ammonium, which is a relatively rapidly convertible N-source, exerts negative effects on both Pen and Ceph biosynthesis (Martín & Aharonowitz, 1983; Nüesch et al., 1987; Sánchez et al., 1988; Demain, 1991; Peñalva et al., 1992; Feng et al., 1994; Haas & Marzluf, 1995; Haas et al., 1995; Brakhage, 1997,1998; Marzluf, 1997b); however, it is frequently used in industrial fermentations as a low-concentration feed (Herschbach et al., 1984; Nielsen, 1995). Phosphate uptake by β-lactam producing filamentous fungi may occur via a system similar to one of the recently characterised H^+-phosphate or Na^+-phosphate symporters of *S. cerevisiae* and *N. crassa* (Bun Ya et al., 1991; André, 1995; Garril, 1995; Versaw, 1995; Versaw & Metzenberg, 1995; Yompakdee et al., 1996) (Fig. 5). Ammonium uptake by *P. chrysogenum* occurs via an apparent uniport system, possibly

in conjunction with passive diffusion (Hackette et al., 1970; Goldsmith et al., 1973). In *S. cerevisiae* three *pmf*-driven ammonium transporters have been characterised (André, 1995; Nelissen et al., 1997). Nitrate uptake has been studied in *A. nidulans*, where it is mediated by the *crnA*-gene product (Brownlee & Arst, 1983; Unkles et al., 1991). Urea uptake in *S. cerevisiae* is thought to be mediated by an active and a facilitated system (André, 1995).

Sulphate is used as the S-source in β-lactam productions (Herschbach et al., 1984; Demain, 1991). The sulphate uptake system in *P. chrysogenum* has been characterised in detail either using mycelium (Tardrew & Johnson, 1958; Segel & Johnson, 1958; Yamamoto & Segel, 1966; Tweedie & Segel, 1970; Bradfield et al., 1970; Cuppoletti & Segel, 1975) or using isolated plasma membranes (Hillenga et al., 1996a). Sulphate is actively taken up in symport with two protons by a high-affinity, *pmf*-dependent system which is induced by sulphate starvation (Fig. 5). At the genetic and biochemical level, highly regulated sulphate permeases have been demonstrated in *A. nidulans* and *N. crassa* (Hussey et al., 1965; Arst, 1968; Marzluf, 1970ab; Ketter & Marzluf, 1988; Ketter et al., 1991; Jarai & Marzluf, 1991; Marzluf, 1997a) which show similarity to sulphate transporters in *S. cerevisiae* (Smith et al., 1995; Cherest et al., 1997; Nelissen et al., 1997).

Some of the mentioned transporters that are characterised at the molecular level, are secondary transporters belonging to the MFS, whereas others are classified differently (Marger & Saier, 1993; André, 1995; Nelissen et al., 1997; Pao et al., 1998; Saier, 1998) (Fig. 5). The transporters of phosphate, nitrate and sulphate (as well as sugars and amino acids) in β-lactam producing filamentous fungi putatively resemble analogous transporters in plants (Tanner & Caspari, 1996; Logan et al., 1997).

Amino acids

Although amino acids are not always specifically supplied to the growth medium in industrial *P. chrysogenum* or *C. acremonium* fermentations, for several reasons the uptake of amino acids is worthwhile to be considered. First, uptake of amino acids may contribute to β-lactam antibiotic production in later growth stages. In these stages the mycelium undergoes fragmentation, e.g., by propelling forces, and autolysis (Paul et al., 1994; Nielsen & Krabben, 1995; Pusztahelyi et al., 1997ab; Schügerl, 1998), whereupon small peptides and single amino acids might be released by the action of proteases and be re-utilised by ongrowing mycelium. Second, the addition to the growth medium of some amino acids enhances β-lactam antibiotic formation. Examples are Glu in the case of Pen production (Lara et al., 1982; Jaklitsch et al., 1985; but see Brakhage, 1998), and Met in the case of Ceph biosynthesis (Demain, 1991; Velasco et al., 1994; Weil et al., 1995). Other amino acids have negative effects on β-lactam antibiotic biosynthesis (Demain, 1991; Brakhage & Turner, 1992; Then Bergh & Brakhage, 1998). Third, special additives to the growth medium may contain specific amino acids. An example is corn steep liquor that is used in *Penicillium* fermentations, which contains Val (Jørgensen et al., 1995b). Fourth, supply of amino acids may become necessary upon engineering primary amino acid metabolism. As an example, lysine had to be added upon breaking up the lysine biosynthetic pathway downstream of Aad (Casqueiro et al., 1998ab; Gutiérrez et al., 1998).

Studies of amino acid transport in fungi, including *A. nidulans* and *P. chrysogenum*, have been reviewed by Horák (1986, 1997), Roos (1989), André (1995), and Sophianopoulou & Diallinas (1995). Mechanistically, it appears that all amino acid uptake in fungi occurs as secondary transport, and that most, if not all, transporters are members of the amino acid–polyamine–choline (APC) family (Nelissen et al., 1997; Paulsen et al., 1998; Saier, 1998). Systems are discriminated by substrate specificity (e.g., hydrophobic, aromatic, acidic, basic, neutral, Cys, Met), affinity, and expression pattern. In *P. chrysogenum*, the ACV precursor Aad is transported (Friedrich & Demain, 1978; López-Nieto et al., 1985; Hönlinger & Kubicek, 1989b) by the acidic amino-acid permease (system IV) (Hunter & Segel, 1971) (Fig. 5), and Val (López-Nieto et al., 1985; Affenzeller & Kubicek, 1991; Hillenga et al., 1994) is transported by the general amino acid permease (system III) (Benko et al., 1969; Hunter & Segel, 1973a) (Fig. 5). Both uptake systems are induced under nutrient-starvation conditions. Cys is transported (López-Nieto et al., 1985) by one of two specific Cys permeases (systems II and IX), of which one is expressed under S-starvation and the other under S-sufficient conditions (Skye & Segel, 1970) (Fig. 5). Recently, the basic amino acid permease of *P. chrysogenum* has been characterised in isolated plasma membrane vesicles (Hillenga et al., 1996b). It was demonstrated that it functions in a reversible manner instead of uni-directionally, and that it is not regulated by *trans*-inhibition. This was in contradiction with the hypothesis that uni-directionality

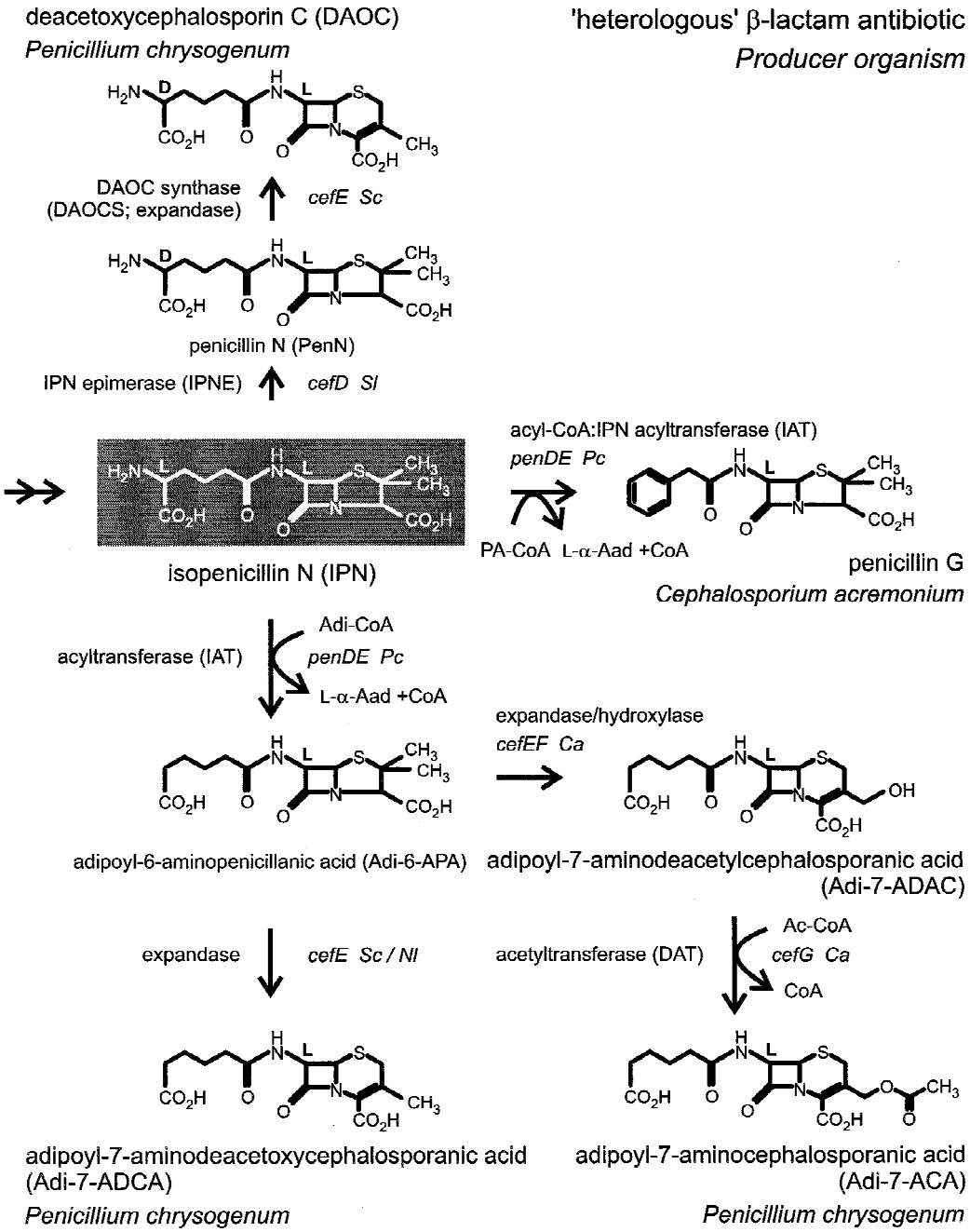

Figure 6. Biosynthetic pathways for 'heterologous' β-lactam antibiotics, obtained by transformation of either *Penicillium chrysogenum* or *Cephalosporium acremonium* with heterologous genes from either *Cephalosporium acremonium* (*Ca*), *Nocardia lactamdurans* (*Nl*), *Penicillium chrysogenum* (*Pc*), *Streptomyces clavuligerus* (*Sc*), or *Streptomyces lipmanii* (*Sl*). The biosynthetic routes are depicted starting with the 'homologous' isopenicillin N.

and *trans*-inhibition are generally encountered regulatory mechanisms in fungal amino acid uptake (Horák, 1986).

Side-chain precursors phenylacetic and phenoxyacetic acid.

The sidechain precursors PA and POA have to cross the plasma membrane. In some reports the presence of

special PA and POA uptake systems in *P. chrysogenum* (Fernández-Cañón et al., 1989ab; Martínez-Blanco et al., 1989; Tan et al., 1993; Luengo, 1995) and in *A. nidulans* (Fernández-Cañón & Luengo, 1997) was indicated, similar to the situation in the Gram-negative bacterium *Pseudomonas putida* (Schleissner et al., 1994). However, recent results have shown that both molecules are equally rapidly and independently taken up by ΔpH-stimulated passive diffusion of the undissociated molecules across the plasma membrane without the need to invoke carrier-mediated transport (Eriksen et al., 1994,1995; Hillenga et al., 1995) (Figs. 2 & 5). Bulk diffusion of PA or POA leads to collapse of the Δp (Henriksen, 1996). This may in part explain the toxic effect of these molecules when present in high amounts or at unfavourably low pH (Barrios-González et al., 1993; Henriksen, 1996).

Penicillin and cephalosporin biosynthesis intermediates.

During Pen biosynthesis, ACV accumulates in the broth but the extracellular IPN concentration remains low throughout the fermentation (Jørgensen et al., 1995b) (*vide supra*, Section 3). The extracellular ACV concentration is about one-third of the intracellular concentration. ACV may be transported out of the cell by the GSH export system (Meister & Anderson, 1983; Schwartz et al., 1988; del Carmen Mateos & Sánchez, 1990) (Figs. 4 and 5) which might resemble the plasma membrane-located ATP-dependent GSX transporter found in higher eukaryotes (Deeley & Cole, 1997; Rea et al., 1998). OPC is efficiently excreted from the cell (Hönlinger & Kubicek, 1989b) by an unknown transporter (Fig. 5). IPN is either converted efficiently by IAT intracellularly (Jørgensen et al., 1995b; *vide supra*, Section 3), or it might be regained via transport across the plasma membrane (Fig. 5). A GSH import system has been shown to develop upon sulphur deficiency in *P. chrysogenum* (Hunter & Segel, 1971) (Figs. 4 and 5). During Ceph biosynthesis, PenN and DAC accumulate in the broth (Fujisawa et al., 1973; Nüesch et al., 1987). Remarkably, whereas a block either in the expansion step, or in the terminal transferase reaction, results in a huge extracellular accumulation of either PenN (Felix et al., 1981) or DAC (Fujisawa et al., 1975; Nüesch et al., 1976), a block in the intermediate hydroxylase reaction leads to intracellular accumulation of DAOC (Traxler et al., 1975). Apparently, PenN and DAC exhibit certain properties that facilitate their secretion, but it is impossible for the cell to excrete DAOC.

Penicillin and cephalosporin

Ceph resembles ACV, IPN, PenN, DAOC and DAC with respect to hydrophilicity. These molecules have two carboxylate groups and one free amino group. It is unclear whether they can leave the cell by passive diffusion. Pen is relatively hydrophobic due to the exchange of the polar Aad sidechain by the less polar P(O)A, and might pass the plasma membrane by passive diffusion. It has been reported that externally added Pen inhibits Pen biosynthesis (Martín et al., 1979; Herschbach et al., 1984), which is explained most easily by diffusion of Pen across the plasma membrane. Apart from these indications, however, not much is known about the mechanism of β-lactam antibiotic excretion by the producing fungal cells.

The fact that β-lactam antibiotics may be administered using liposomes as drug-delivery vesicles has triggered studies of the permeability of phospholipid membranes of various compositions. This provides with some indications concerning excretion by the producers. For example, β-lactam antibiotics have been shown to freely and rapidly permeate phospholipid bilayers of liposomes composed of *E. coli* phospholipids. Permeability was proportional to drug hydrophobicity: Pens permeate with higher efficiency than the more hydrophilic Cephs (Yamaguchi et al., 1982). Lipopolysaccharides had a negative effect on the permeability of these liposomes (Hiruma et al., 1984). Pen lowers the viscosity of phosphatidylcholine containing liposomes (Kirschbaum, 1986; Suwalsky et al., 1996). Liposomes made of egg phosphatidylcholine:cholesterol:diacetylphosphate (80:20:1) appeared to be less permeable for β-lactam antibiotics than liposomes made of rat intestinal lipids (Kimura et al., 1980). An increasing amount of negatively charged dipalmitoylphosphatidylserine, and an increasing surface potential, exert negative effects on the diffusion of anionic (but not of zwitterionic) Cephs through phospholipid bilayers (Sugawara et al., 1994ab).

Taken together, β-lactam antibiotics might be able to leave the producing cell via passive diffusion. However, there are several reasons to invoke a special Pen transport system or pathway for the export across the plasma membrane (Fig. 5). First, negative factors such as tight lipid packing due to the phospholipid and ergosterol content of the *P. chrysogenum* plasma membrane, and surface and electrochemical membrane potentials, are likely to drastically inhibit passive diffusion of Pens and Cephs (*vide supra*, and Hillenga et al., 1994, and unpublished). Second, passive diffu-

sion can not account for a Pen distribution with higher concentrations outside than inside the cell (*vide supra*, Section 3). That is, when Pen (pK_a=2.73) would be distributed according to the ΔpH across the plasma membrane, its distribution would be inverted.

Fungal β-lactam antibiotic excretion could be mediated by systems which resemble active primary (ATP-driven) or secondary (*pmf*-driven) transport systems that are involved, e.g., in β-lactam antibiotic transport across heterologous membranes as exemplified by the H^+-oligopeptide transporters of intestinal and renal brush-border membranes (Kramer et al., 1992; Ganapathy et al., 1995; Daniel, 1996; Naasani et al., 1996; Döring et al., 1998; Iseki et al., 1998) and by complex bacterial efflux pumps (Ma et al., 1994; Nikaido, 1994; Poole et al., 1996; Koehler et al., 1997; Srikumar et al., 1997; Nikaido et al., 1998), in the excretion of bacterial antibiotics (Cundliffe, 1989; Coque et al., 1993a; Nikaido, 1994; Méndez & Salas, 1998), or in multidrug resistance (Higgins, 1992; van Veen & Konings, 1997; Del Sorbo et al., 1997; Tobin et al., 1997; Nakaune et al., 1998). As a third option next to passive diffusion and protein-mediated active transport, vesicular transport (Luengo et al., 1986; Kuryłowicz et al., 1987; Kurzątkowski & Kuryłowicz, 1991; Müller, 1991) can not be excluded. Vesicular transport of solutes is involved in, e.g., cell wall synthesis, neurotransmitter release, pheromone secretion, transport and excretion of bile acids, and the expelling of GSXes, and occurs in all kinds of eukaryotic organisms (Schekman, 1992; Bennet & Scheller, 1993; Brodelius & Pedersen, 1993; Leal-Morales et al., 1994; Kolling & Hollenberg, 1996; Pierre et al., 1994; Sollner & Rothman, 1994; Liu et al., 1995; Meier, 1995; Kühn et al., 1996; Moriyama et al., 1996; Regalado et al., 1997).

Pens are eventually produced inside microbodies which opens up the possibility of vesicle-mediated transport between microbodies and the plasma membrane, thereby circumventing free passage through the cytosol (Fig. 5). On the other hand, Cephs and 'heterologous' β-lactam antibiotics, also those produced by *P. chrysogenum* (*vide infra*, Section 5), are synthesized in the cytosol. This would mean, either that they use a different route than Pens, or that all β-lactam antibiotics could be expelled from the cytosol via uptake into secretory vesicles and subsequent fusion of these vesicles with the plasma membrane (Fig. 5). It has been suggested that the microbody membrane is purportedly more permeable to Pens than the plasma membrane due to its different phospholipid, sterol and protein composition (*vide supra et infra*). Passage of Pen through the cytosol may in part explain the formation of 6-APA which has been considered to be due to cytosolic acylase activity (Nielsen, 1995).

4.2 Transport across the vacuolar membrane

The fungal vacuole is surrounded by a single, impermeable membrane. It serves as a storage compartment, and is involved in, e.g., ion and pH homeostasis, detoxification and protein degradation inside the fungal cell (Klionsky et al., 1990; Jones et al., 1997). Important metabolites stored inside the vacuole are, e.g., basic amino acids, phosphate (stored as polyphosphate), sulphate and calcium (Klionsky et al., 1990; Lendenfeld et al., 1993; Jones et al., 1997). A specific role in sulphur amino acid homeostasis has been shown (Elskens et al., 1991; Jacquemin-Faure et al., 1994; Mehdi & Penninckx 1997). The vacuolar pH is lower than the cytosolic pH, namely 5-6 versus approx. 7.0, and is maintained by the concerted action of a V-type (V_0V_1-type) H^+-ATPase which pumps protons into the vacuole at the expense of ATP (Fig. 5), a potassium cation channel and a chloride anion transporter, resulting in a ΔpH and $\Delta\psi$ (acidic, positive inside) of considerable magnitude (Kakinuma et al., 1981; Bowman & Bowman, 1982,1986; Wada et al., 1987; Harvey & Nelson, 1992; Hillenga et al., 1994; Wada & Anraku, 1994; Anraku, 1996).

The mechanism of transport of small solutes such as amino acids across the vacuolar membrane is probably antiport with protons or other amino acids (for import) and symport with protons or antiport with other amino acids (for export), as demonstrated in *S. cerevisiae* (Sato et al., 1984ab; Klionsky et al., 1990; Jones et al., 1997). Isolated vacuoles from *P. chrysogenum* have the capability to import Lys, Aad and Val (Lendenfeld et al., 1993) (Fig. 5). Control over the vacuolar amino acid pools appears to be exerted by cytosolic adenylates, and cytosolic amino acid concentrations (Kitamoto et al., 1987; Roos et al., 1997). GSH and its conjugates (as well as ACV and *bis*-ACV, possibly) are generally transported via ATP-dependent GSX transporters (Figs. 4 & 5). This class of proteins is widely encountered amongst eukaryotes, e.g., in yeast (Szczypka et al., 1994; Wemmie et al., 1994; Cui et al., 1996; Li et al., 1996,1997), man (Ishikawa 1992; Deeley & Cole, 1997), plants (Martinoia et al., 1993; Li et al., 1995; Hell, 1997; Lu et al., 1998; Rea et al., 1998), and protozoan parasites (Grondin et al., 1997). The mechanism of sulphate transport across the

vacuolar membrane (Hunter & Segel, 1985), which also takes place in plants (Hell, 1997), is unknown.

During differentiation of the mycelium of *P. chrysogenum*, a development is seen in the size and number of vacuoles (Paul et al., 1994; Nielsen, 1995; Vanhoutte et al., 1995; Paul & Thomas, 1996). Vacuoles in apical hyphal compartments are small, but they increase in size and number in subapical regions of the mycelium. In the 'heart' of mycelial pellets, which are hardly accessible from the outside and thus suffer from a lack of supply of, e.g., energy sources and oxygen, vacuoles are involved in autolytic and auto-degradative processes (Paul & Thomas, 1996; van den Hazel et al., 1996; Pusztahelyi et al., 1997ab). Fragmentation of the mycelium occurs preferentially in regions with heavy subapical vacuolation (Paul et al., 1994). A positive correlation has been drawn between the extent of this type of vacuolation and Pen production in *P. chrysogenum*, but the exact meaning of this correlation is uncertain (Paul & Thomas, 1996).

4.3 Transport across the microbody membrane

Microbodies is the collective term for 0.1–1 μm diameter, specialised, often inducible, organelles, comprising, e.g., peroxisomes and glyoxysomes, surrounded by a single membrane. They are involved in various metabolic pathways in different organisms, e.g., H_2O_2 metabolism, β-oxidation of fatty acids and dicarboxylates, and the metabolism of glyoxylate, cholesterol and ether lipids (Osmundsen et al., 1991; de Hoop & AB, 1992; van den Bosch et al., 1992; Sulter et al., 1993a; Lazarow & Kunau, 1997; Waterham & Cregg, 1997). Data on their membrane properties are ambiguous (Lazarow & Kunau, 1997). Reports indicate that they may have an acidic interior, maintained by the action of a H^+-ATPase (Douma et al., 1987; Nicolay et al., 1987). This seems hardly compatible with the reported *in vitro* permeability of peroxisomes (Van Veldhoven et al., 1987; Douma et al., 1990; Heupel et al., 1990; Sulter et al., 1993a; Heupel & Heldt, 1994; Reumann et al., 1994). The permeability has been ascribed to the presence of (regulated) porins in their membranes (Van Veldhoven et al., 1987; Sulter et al., 1993b; Reumann et al., 1995). Recent *in vivo* studies show that peroxisomal membranes are impermeable for small solutes such as NAD(P)H, acetyl-CoA and activated fatty acids (Singh et al., 1992; van Roermund et al., 1995; Tabak et al., 1995; Hettema et al., 1996), invoking specialised transporters. A few of such small-solute transporters, most of them belonging to the ABC superfamily of primary transporters (Higgins, 1982; Decottignies & Goffeau, 1997; Saier, 1998), have recently been described (Kamijo et al., 1990; Jank et al., 1993; Mosser et al., 1993; McCammon et al., 1994; Cartier et al., 1995; Elgersma et al., 1995; Shani et al., 1995,1996; Hettema et al., 1996; Lombard-Platet et al., 1996; Sakai et al., 1996; Swartzman et al., 1996; Shani & Valle, 1998). As a final note, active transport of small solutes occurs in parallel with the import by a multi-component membrane-located protein import machinery of matrix proteins that are putatively in their folded state (Subramani, 1993,1996; Rachubinski & Subramani, 1995; Waterham & Cregg, 1996; McNew & Goodman, 1996; Elgersma & Tabak, 1996). Thus, *in vivo* impermeability for relatively small solutes is a property of the peroxisomal membrane which appears to occur alongside with the ability to accommodate the passage of bulky-sized proteins.

Microbodies have been encountered in various filamentous fungi (Maxwell et al., 1975; Carson & Cooney, 1990), including *P. chrysogenum* (Müller et al., 1991,1995), *A. nidulans* (Valenciano et al., 1996,1998; De Lucas et al., 1997,1998), *A. niger* (van Dijken & Veenhuis, 1980; Schilling & Lerch, 1995), *N. crassa* (Wanner & Theimer, 1982; Kionka & Kunau, 1985; Thieringer & Kunau, 1991ab; de Zoysa & Connerton, 1994), and *Podospora anserina* (Berteaux-Lecellier et al., 1995). In these organisms, they are involved in diverse functions such as β-oxidation, Pen biosynthesis, and sexual development. Growth of *A. nidulans* under Pen-producing conditions resulted in a moderate proliferation of microbodies (Valenciano et al., 1998). A mutant of *A. nidulans* defective in microbody biogenesis, which lacked functional microbodies, was not impaired in Pen production, however (Valenciano et al., 1996; De Lucas et al., 1997,1998). This suggests that microbodies are not essential for Pen biosynthesis. On the other hand, when the signal was deleted which targets IAT to the microbody in *P. chrysogenum*, no Pen production was observed (Müller et al., 1992). This may indicate that the microbodies contain another enzyme which is essential for Pen production, possibly PCL (*vide supra*, Section 2.5). The alkaline pH optima of IAT (Alvarez et al., 1987,1993) and PCL (International patent WO97/02349) suggest the microbody interior to be alkaline, unlike what has been found for yeasts (Douma et al., 1987; Nicolay et al., 1987).

No data are available on the permeability of the microbody membrane for P(O)A, CoA and ATP, which

are the substrates for PCL, or for P(O)A-CoA and IPN, which are the substrates for IAT, or Pen and Aad, which are produced by the IAT-catalysed reaction (Figs. 1–3 & 5). In view of the permeability properties of P(O)A (Hillenga et al., 1995), it is likely that P(O)A diffuses freely across the microbody membrane (Fig. 5). The generally observed low sterol content of the microbody membrane (Sulter et al., 1993a; Zinser & Daum, 1995) may yield it permeable for IPN and Pen. Active transport of CoA (or P(O)A-CoA) and ATP is possibly requested, however. This may involve shuttles that are similar to those encountered in the mitochondrial inner membrane (Jank et al., 1993; Kuan et al., 1993; Elgersma et al., 1995; van Roermund et al., 1995; Tabak et al., 1995; Sakai et al., 1996).

5. Compartmentalization and transport in biotechnologically engineered 'heterologous' β-lactam antibiotic biosynthesis

Inspired by the generally higher antibiotic-producing capacity of *P. chrysogenum* relative to *C. acremonium*, and the higher profits of Cephs at concomittant higher costs for *Cephalosporium* fermentations, efforts have been made to use *P. chrysogenum* as a producer of expanded antibiotics (Skatrud, 1992; Díez et al., 1997; Nielsen, 1998). To that end, *P. chrysogenum* was transformed with the following (combinations of) heterologous genes yielding the corresponding 'heterologous' β-lactam antibiotics: (1) IPNE-encoding *cefD* from *Streptomyces lipmanii* and expandase-encoding *cefE* from *S. clavuligerus*, yielding DAOC (Cantwell et al., 1992) (Figs. 6 and 7); (2) expandase-encoding *cefE* from *S. clavuligerus* without *cefD*, yielding DAOC along with 16-hydroxyadipoyl-7-ADCA (Alvi et al., 1995); (3) expandase-encoding *cefE* from *S. clavuligerus* together with a feed of adipic acid (Adi), yielding adipoyl-7-ADCA (Crawford et al., 1995) (Figs. 6 and 7), (4) expandase/hydroxylase-encoding *cefEF* from *C. acremonium* together with an Adi feed, yielding adipoyl-7-ADAC (Crawford et al., 1995) (Figs. 6 and 7), (5) expandase/hydroxylase-encoding *cefEF* and DAT-encoding *cefG* from *C. acremonium* together with an Adi feed, yielding adipoyl-7-ACA (Crawford et al., 1995) (Figs. 6 and 7), and (6) expandase-encoding *cefE* from *S. clavuligerus* or *Nocardia lactamdurans* (*N. lactamdurans*) together with an Adi feed, yielding adipoyl-7-ADCA (Bovenberg et al., 1998) (Figs. 6 and 7).

The *C. acremonium* expandase/hydroxylase and DAT probably have a cytosolic location (*vide supra*; Section 2), but the *S. clavuligerus* expandase (Kovacevic et al., 1989) not the *N. lactamdurans* expandase (Coque et al., 1993b) may end up in the microbody due to the fortuitous presence of a PTS1 signal at its C-terminus (de Hoop & AB, 1992; Elgersma et al., 1996) (Fig. 7). There seemed to be no effect of a putatively different location of the heterologous expandase on adipoyl-7-ADCA production; although the strain with the *N. lactamdurans* expandase showed lower adipoyl-7-ADCA titers, this was attributed to a lower activity of the expandase (Bovenberg et al., 1998). All 'heterologous' β-lactam antibiotics were found in the culture broth. This implies that they are excreted, irrespective whether they were synthesized in the cytosol or in the microbody. The apparent finding that the bacterial expandases accept adipoyl-6-APA and IPN as substrates, is in contradiction with earlier reports about their substrate range (Yeh et al., 1991; Maeda et al., 1995).

The mechanism of uptake of Adi, which was used for the production of adipoyl-7-ADCA, -ADAC and -ACA, across the plasma membrane (Fig. 7) is unknown. Adi is not a substrate for the acidic amino acid carrier of *P. chrysogenum* (van de Kamp et al., unpublished). It might be taken up via a plasma membrane located dicarboxylate transporter (Corte-Real & Leao, 1990; Cassio & Leao, 1991,1993; Sousa et al., 1992; Grobler et al., 1995). It is unknown whether Adi is a substrate of the cytosolic ACS or of the microbody-located PCL in *P. chrysogenum*.

The production of Pens by Ceph-producing *C. acremonium*, has been achieved by transforming it with the IAT-encoding *penDE* gene (plus the IPNS-encoding *pcbC* gene) from *P. chrysogenum* (Fig. 6). When fed with PA, such transformants did yield PenG (Gutíerrez et al., 1991b) (Fig. 7). Interestingly, whereas in Ceph biosynthesis the microbody is disregarded, Pen biosynthesis invokes the microbody for its side-chain exchange (Fig. 2). Apparently, the different cell biology of both biosynthetic processes does not impair Pen biosynthesis by *C. acremonium* which is transformed with the *penDE* gene solely. So, at least in the case of *C. acremonium*, all putative auxiliary functions (e.g., PA-CoA ligases and transport enzymes) appear to be undedicated functions of the fungal cell which are available at the request of Pen biosynthesis. This might more generally be the case amongst filamentous fungi, since the sole introduction of the Pen biosynthesis gene cluster in *N. crassa* and *A. niger*

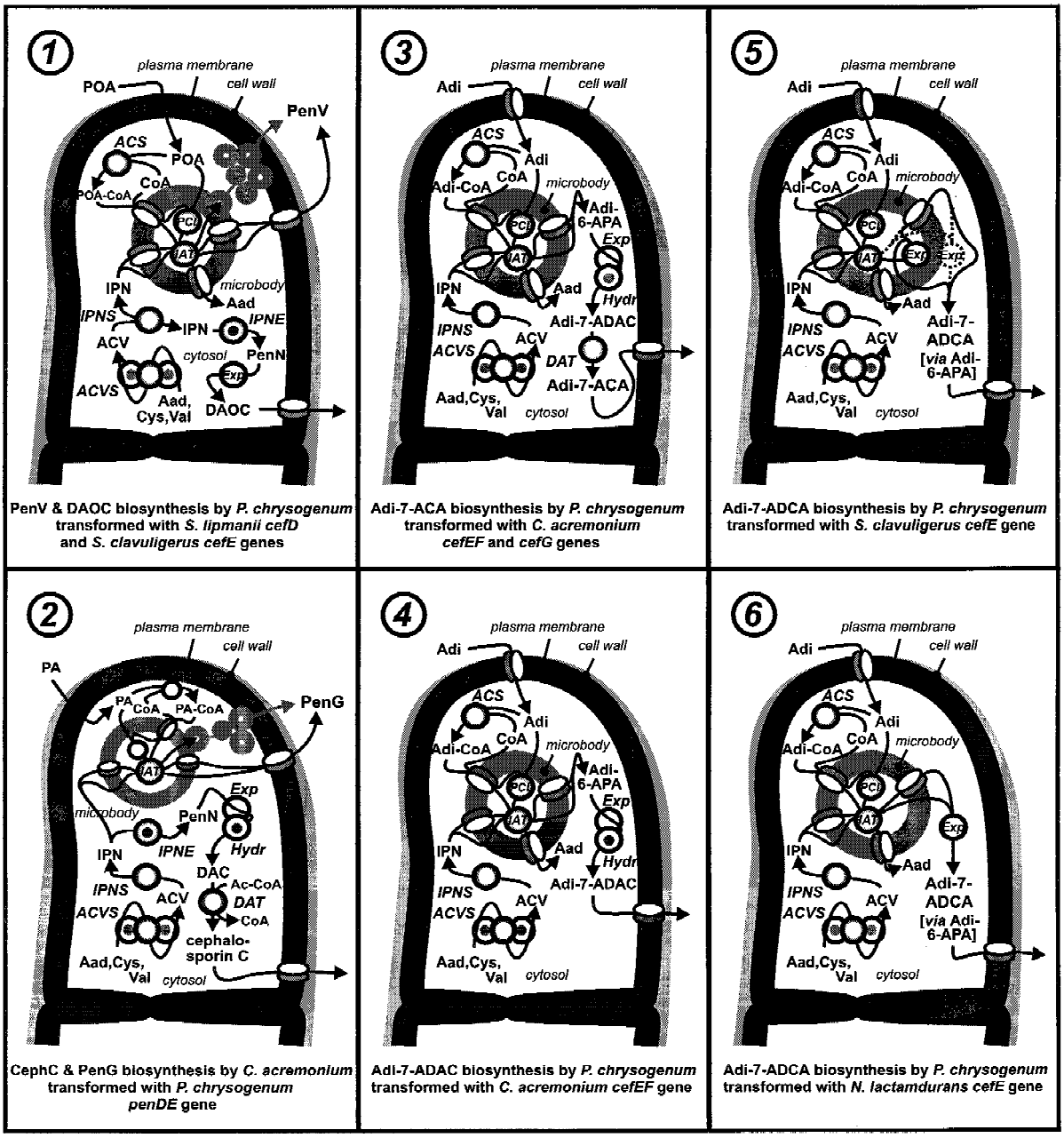

Figure 7. Models depicting the compartmentalization of biotechnologically engineered 'heterologous' β-lactam antibiotic biosynthetic pathways (see Fig. 6 and text). Some putative enzymatic and transport steps are depicted as parallel routes.

also resulted in Pen production, although at a low level (Smith et al., 1990a). Whether *P. chrysogenum* and *C. acremonium* do have exclusively developed auxiliary functions available for the production of Pens and Cephs, respectively, is unclear yet.

6. Concluding remarks and perspectives

Despite recent progress, our knowledge about the importance of intracellular compartmentalization of fungal β-lactam biosynthesis is still limited. On the one hand, organellar compartmentalization of enzymatic steps does not seem to be absolutely necessary for

β-lactam antibiotic biosynthesis. On the other hand, a positive correlation has been observed between the number of microbodies and Pen production (Müller et al., 1991; Valenciano et al., 1998), as well as between the extent of vacuolation and Pen biosynthesis (Paul & Thomas, 1996). In order to trace a direct significance and to obtain direct proof, manipulation of either enzyme locations, organelle characteristics, or transport processes is needed.

The excretion of fungal β-lactam antibiotics is a very interesting but barely touched research area. Elucidation of the transport mechanism and determination of substrate range are important goals in this field. Presently, all different kinds of processes can be envisaged. The final products may be imported from the cytosol into secretory vesicles, which deliver their content to the medium by fusion with the plasma membrane. Alternatively, transport may occur directly across the purportedly relatively β-lactam-impermeable plasma membrane. Such putatively low-affinity carrier-dependent transport events (import in vesicles and secretion across the plasma membrane) across the plasma membrane may be mediated, either by *pmf*-driven secondary transporters or by ATP-dependent primary transporters. As a third option, vesicle-mediated transport from microbody to plasma membrane may occur in the case of Pen production. Although not impossible, such a process does not seem to be invoked by necessity, since the microbody membrane is putatively permeable for Pen. Moreover, it is not directly conceivable that Pen excretion would involve a route that is completely different from Ceph excretion.

In terms of biotechnological innovation, compartmentalization and transport impose boundary conditions that restrict the physiological feasibility of genetically engineerable constructs. Two possible innovative approaches are, e.g., the use of alternative side-chain precursors for cost-effective *in vivo* synthesis of new Pens and Cephs (e.g., Luengo, 1995; Bovenberg et al., 1998), and the *in vivo* biosynthesis of hybrid peptide antibiotics by knowledge-based engineering of ACVS (e.g., Marahiel et al., 1997). This imposes the need to engineer the whole follow-up enzymology in order to congruently modulate their substrate specificity. Importantly, transport steps which are involved in uptake, transport across organellar membranes, and excretion, have to be reconsidered too. In order to make such an extensive pathway-revision possible, the elaboration upon our current understanding of fungal β-lactam antibiotic biosynthetic enzyme structure-function relationship, and of various transport processes, will be a formidable task for the nearby future.

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